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Patentanmeldung Nr. Patent application No. Demande de brevet n°

02079665.2

Der Präsident des Europäischen Patentamts:
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk

Anmeldung Nr:
Application no.: 02079665.2
Demande no:

Anmeldetag:
Date of filing: 07.11.02
Date de dépôt:

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
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C-type lectin binding molecules, identification and uses thereof

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

A61K31/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR IE IT LI LU MC NL PT SE SK TR

Title: C-type lectin binding molecules, identification and uses thereof.

The invention relates to the field of immunology. The invention in particular relates to the role of dendritic cells in immune responses and to pathogens that are able to capitalize thereon.

Dendritic cells (DC) are professional antigen presenting cells (APC) that induce cellular immunity upon pathogen recognition. These cells are therefore important in the defense against many pathogens [1;2;3]. Immature DC are seeded throughout peripheral tissues to act as sentinels against invading pathogens[4]. Upon pathogen capture, DC are activated, process pathogens for antigen presentation on Major Histocompatability Complex (MHC) class II molecules, and migrate to the secondary lymphoid organs where they activate naïve T cells to initiate adaptive immune responses[1;3;4]. Depending on the pathogen that is recognized by the DC, differentiation of naïve T cells into Th1 cells is triggered by DC in response to intracellular microbes, whereas Th2-mediated responses are generated by DC to eliminate pathogens residing extracellularly [3]. Thus DC play an important role in both innate and cellular immune responses against tumors antigens as well as pathogens such as viral, bacterial, fungal and parasitic infections[1;5]. Knowledge about cell-surface receptors on DC that are involved in recognition of pathogens is only starting to emerge, and include Toll-like receptors (TLR) [6;7] and C-type lectins [8]. TLR recognize specific pathogen-derived components, such as lipoproteins, lipopolysaccharides and bacterial DNA, and relay this information through intracellular signaling cascades leading to the production of regulatory cytokines and upregulation of MHC and costimulatory molecules that lead to activation/maturation of DC[6]. In contrast, C-type lectins recognize pathogen-derived carbohydrate structures and upon binding internalize pathogens for antigen processing and presentation to T cells[8-10]. In classical calcium-dependent lectins, conserved amino acid residues in the carbohydrate

recognition domain (CRD) are involved in calcium binding and sugar specificity [11]. A growing number of C-type lectins are described to be specifically expressed by DC. For most of these lectins detailed knowledge about pathogen-targets as well as cellular ligands if any, including the identity of the carbohydrate structure they recognize, is lacking[8]. The DC-specific C-type lectin DC-SIGN (dendritic-cell specific ICAM-3 grabbing nonintegrin, CD209) is involved in binding of the HIV-1 envelope glycoprotein by DC to enhance infection of T cells [12], while the mannose receptor (MR) is involved in recognition of mycobacteria and Fungi/Protozoa [13]. Some C-type lectins like DC-SIGN can interact with carbohydrate-bearing self glycoproteins (ICAM-2 and ICAM-3) to mediate cellular adhesion processes [14;15].

In one aspect of the present invention it was found that a C-type lectin can comprise specificity for more than one type of glycoconjugate. This knowledge is useful for a variety of purposes. For instance, lectins are instrumental in mediating pathogen binding and the presentation of antigens thereof in dendritic cells. This binding property of C-type lectins is utilized by a number of pathogens to at least in part facilitate infection of an individual. Knowledge of glycoconjugate specificity of such lectins therefore provides an entrance to the development of medicaments capable of interfering with the capacity of carbohydrates present on pathogens to interact with the lectin and thereby at least in part interfere with the infection or the severity thereof in the exposed individual. In one embodiment the invention therefore provides a method for at least in part inhibiting the binding of a ligand to a C-type lectin or a carbohydrate binding part thereof, comprising contacting said C-type lectin with an isolated and/or recombinant glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a derivative or multimer thereof. Also provided is the use of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a derivative or multimer thereof

for at least in part inhibiting the binding of a ligand to a C-type lectin or a carbohydrate-binding part thereof. This method may be used for instance to study the exact binding properties of the lectin. The method is also of use in identifying compounds capable of interfering with an ability of said C-type lectin to bind to pathogens. In a preferred embodiment said method further comprises a cell comprising said C-type lectin. Preferably said cell is an antigen presenting cell, more preferably a dendritic cell or macrophage. In the presence of such cell, the effect of such inhibition may be compared to the function of the antigen presenting cell, for instance on its capacity to present antigen and/or the production of lymphokines and/or cytokines. In a preferred embodiment said C-type lectin comprises DC-SIGN, L-SIGN and/or DC-SIGNR. Whereas DC-SIGNR and L-SIGN are predominantly expressed on macrophage/endothelial cells, on lymph node and on liver sinusoidal endothelial cells and DC-SIGN is expressed predominantly on dendritic cells. DC-SIGNR and L-SIGN share the remarkable binding and signaling effects that ManLAM has upon binding to dendritic cells via DC-SIGN. However, whereas DC-SIGN further comprises a particular specificity for glyconjugates comprising a fucose residue or a derivative or multimer thereof, DC-SIGNR and L-SIGN are lacking such particular specificity. In the present invention there is thus a preference for the C-type lectin DC-SIGN.

With the term "glycoconjugate comprising a fucose residue" is meant a glycoconjugate comprising at least one fucose residue that is α1, 3 or α-1,4-linked to the glycoconjugate. The linking moiety preferably consists of N-acetylglucosamine. The linking moiety is preferably coupled to an oligosaccharide or glycan, which in turn may be part of a larger structure (or carrier molecule) comprising subsequent glycoconjugates comprising a fucose residues, or other compounds such as mannose in any configuration. A derivative of said glycoconjugate comprising a fucose residue comprises the same C-type lectin binding activity in kind not necessarily in amount. A

derivative may be generated through modification of the fucose residue. Such modifications may be generated in various ways. Preferably, said fucose is a terminal fucose, i.e. linked via only one α1, 3 or α-1,4 linkage to another molecule or fucose. Preferred examples of glycoconjugates comprising a fucose residue are Lewis bloodgroup antigens Le^x, Le^y, Le^a, Le^b and/or LDNF. C-type lectin binding parts, derivatives and/or analogous of the lewis bloodgroup antigens are of course also within the scope of the invention. Different modifications have different effects on the lectin binding properties. Sialylation of Le^x (yielding sialyl-Le^x, a L-, E- and P-selectin ligand) completely abrogates the recognition by DC-SIGN, indicating that DC-SIGN has a carbohydrate specificity that is distinct from that of the selectins that mediate leukocyte rolling. Sulfation reduces the binding affinity of DC-SIGN for Le^x, as well as Le^a (Figure 2a). A derivative of a glycoconjugate comprising a fucose residue thus comprises a modification wherein said modification allows binding of said glycoconjugate to said C-type lectin. Glycoconjugates comprising multiple fucose residues in the configuration of a lewis bloodgroup antigen typically have a higher binding affinity than a glycoconjugate comprising a single fucose residue.

With the term "glycoconjugate comprising at least two mannose residues in α1,2 linkage" is meant a glycoconjugate comprising at least two mannose residues wherein said mannose residues are linked to each other via an α1,2 linkage and wherein one of the mannose residues is linked to the glycoconjugate. The linking moiety preferably but not necessarily comprises mannose in α-1,5 linkage and arabinan or β-D-arabinose. The linking moiety is preferably coupled to an oligosaccharide or glycan, which in turn may be part of a larger structure (or carrier molecule) comprising subsequent glycoconjugates comprising at least two mannose residues in α1,2 linkage, or other compounds such as fucose in any configuration. A derivative of said glycoconjugate comprising at least two mannose residues in α1,2 linkage

comprises the same C-type lectin binding activity in kind not necessarily in amount. A derivative may be generated through modification of one or both of the mannose residues. Such modifications may be generated in various ways. Preferably, said at least two mannose residues in α1,2 linkage are terminal residues, i.e. linked via only linkage to another molecule or mannose. In a preferred embodiment glycoconjugate comprising at least two mannose residues in α1,2 linkage, comprises two or three mannose residues linked to each other via respectively one and two α1,2 linkages. A particularly preferred example of a of glycoconjugates comprising at least two mannose residues in α1,2 linkage is ManLAM. ManLAM comprises a mannose-capped lipoarabinomannan, such as derivable from the cell wall component of Mycobacterium. The mannose-capped lipoarabinomannan is preferably linked to an oligosaccharide or glycan, which in turn may be part of a larger structure (or carrier molecule) comprising subsequent mannose-capped lipoarabinomannans or other compounds such as fucose in any configuration. C-type lectin binding parts, derivatives and/or analogous of ManLAM are of course also within the scope of the invention. Different modifications have different effects on the lectin binding properties. Glycoconjugates comprising two or more sets of at least two mannose residues in α1,2 linkage typically have a higher binding affinity than a glycoconjugate comprising only one set of at least two mannose residues in α1,2 linkage.

Carrier molecules may further comprise a large variety of different molecules including peptide, protein, lipid, polysaccharide and also synthetic molecules not expressly mentioned here. The carrier molecule(s) may of course be part of an even larger structure such as a biological or artificial surface, a virus, a bacterium or a eukaryotic cell.

Binding of the ligand to the C-type lectin can be inhibited by interfering with binding site on the ligand, with binding site of the C-type lectin or both.

Interference with the binding site of the ligand can be done using a ligand binding molecule capable of specifically binding to the C-type lectin binding part on the ligand. This is preferably done with a proteinaceous molecule such as a carbohydrate specific antibody preferably specific for a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glyconjugates comprising a fucose residue or a derivative or multimer thereof. The experimental part describes non-limiting examples of such antibodies. Non-limiting examples of antibodies comprising a specificity for a glyconjugates comprising a fucose residue or a derivative or multimer thereof are SMLDN1.1 or SMFG4.1, 6H3 or SMLDN1.1. The ligand binding molecule can also be a C-type lectin, preferably a soluble derivative thereof, comprising specificity for a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glyconjugates comprising a fucose residue or a derivative or multimer thereof. A non-limiting example of a soluble C-type lectin of the invention is a soluble DC-SIGN-Fc chimeric molecule comprising amino acid residues 64-404 fused at the C-terminus to a human IgG1-Fc fragment as described for instance in Geijtenbeek et al (Geijtenbeek et al 2002, J. Biol. Chem. 277:11314-11320). However, soluble C-type lectins comprising one or more of the provided specificities can be generated in various ways (for instance by derivation from DC-SIGNR or L-SIGN). Now that the present invention describes one chimeric molecule others can be generated, for instance as described in Fawcett J et al (Fawcett J et al 1992, Nature 360:481-4).

Interference with the binding site on the C-type lectin can of course also be done in various ways. Interference with the binding site on the C-type lectin can be done using a C-type lectin binding molecule capable of specifically binding to the ligand binding part on the C-type lectin. This is preferably done with a proteinaceous molecule such as a C-type lectin specific antibody or with a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a derivative or multimer thereof, wherein said glycoconjugate or both may be part of a larger structure.

Binding can of course further be inhibited by sterically hindering the binding of the ligand to the C-type lectin. For this it is not absolutely required that the interfering molecule itself binds to the association site of the carbohydrate and the C-type lectin. Binding can also be interfered with successfully steric hindrance by binding of the interfering molecule in the vicinity of the site of association.

The ligand may be any type of structure capable of binding to said C-type lectin. Usually this will comprise a proteinaceous molecule or a carbohydrate. The ligand may be an antibody. It is preferred, but not strictly necessary, that the ligand comprises mannosilated glycans such as cell wall component of Mycobacterium ManLAM, or a mannose derivative, lipophosphoglycan such as derived from Leishmania, or a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue such as SEA or CD66 or a derivative or multimer thereof. The ligand can have instead of, or in addition to, a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glyconjugate comprising a fucose or a derivative or multimer thereof, different glyconjugates capable of binding to the same on the C-type lectin.

The ligand preferably comprises an antigen. The ligand preferably comprises a (tumor) antigen, a pathogen and/or a cell associated receptor. An antigen is here used to include peptides or glycolipids and derivatives thereof capable of being presented in the context of MHC class I or class II, or CD1b. At least some C-type lectins, and particularly those expressed on dendritic cells are involved in the process of antigen uptake and presentation thereof by antigen presenting cells. An early step in this process is the capture of the antigen by such lectins. With a method of the invention it is possible to at least in part interfere with this initial step in the process of antigen presentation. Considering the importance of antigen presentation in the initiation and maintenance of antigen specific immune responses, it is clear that the method

of the invention can be favorably used to at least in part diminish the potency of an immune response against said antigen.

In a particularly preferred embodiment the ligand comprises a pathogen or a C-type lectin binding part thereof. It has been found that many different pathogens comprise carbohydrates capable of binding to C-type lectins of the invention. In particular it has been found that pathogens or parts thereof can bind to said C-type lectins through a mannose containing glycoconjugate such as a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glyconjugates comprising a fucose or derivative or multimer thereof. This binding at least in part facilitates infection of an individual with said pathogen. Interference with the binding of the pathogen or part thereof to the C-type lectin can thus at least in part aid an individual in combating an infection by the pathogen and in cases even prevent the establishment of a clinically visible symptoms thereof. The latter is useful in (passive or active) vaccination strategies. The pathogen can be a virus, a fungus, a (myco)bacterium and/or a parasite. The pathogen preferably comprises a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a derivative or multimer thereof. Examples of preferred pathogens are human immunodeficiency virus, a mycobacterial infection, a helicobacter, a *leishmania*, a *schistosoma*, a *klebsiella*, a herpes simplex virus or an ebola virus. Inhibition of binding of the specifically mentioned pathogens can be achieved using a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a derivative or multimer thereof. In a particularly preferred embodiment inhibition of binding is achieved using a ligand (pathogen or part thereof) binding molecule capable of specifically binding to the C-type lectin binding part on the ligand. This is preferably done with a proteinaceous molecule such as a carbohydrate specific antibody preferably specific for a mannose carbohydrate such as a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glyconjugate

comprising a fucose residue or a derivative or multimer thereof. The experimental part describes non-limiting examples of such antibodies. The ligand binding molecule can also be a C-type lectin, preferably a soluble derivative thereof, comprising specificity for a mannose carbohydrate such as a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glyconjugates comprising a fucose residue or a derivative or multimer thereof. A non-limiting example of a soluble C-type lectin of the invention is a soluble DC-SIGN-Fc as mentioned above. Thus infections of an individual with said pathogens can at least in part be prevented or treated by administering to said individual a mannose carbohydrate such as a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glyconjugates comprising a fucose residue or a derivative or multimer thereof. In a preferred embodiment a ligand binding molecule of the invention is administered to said individual. This embodiment can also be used to at least in part inhibit the binding of the pathogen to a C-type lectin expressing cell thereby at least in part inhibiting the contamination or spread of the pathogen in the body of a patient, as well as inhibiting DC maturation and cell adhesion, as is also described below.

C-type lectins not only recognize carbohydrate profiles on pathogens but also interact with self glycoproteins to mediate cellular processes such as differentation and migration. A method of the invention can therefore also be used to interfere with the interaction with one or more self-glycoproteins and thereby be used to at least in part inhibit the cellular processes that the mentioned C-type lectins are involved in. This can be done by providing the carbohydrates structures provided by the invention (i.e. mannose containing glycojugate such as a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or derivative or multimer thereof). Preferably, this is achieved using a ligand binding molecule of the invention. Thus in another preferred embodiment the method is used to at least in part inhibit binding of said C-type lectin to a self-glycoprotein

preferably a receptor present on the outer membrane of a cell. Preferably said receptor comprises ICAM-2, ICAM-3, CD166 or CD66 or a functional part, derivative and/or analogue thereof. CD166 and CD66 are present on a subset of NK cells and granulocytes. Thus interaction of these cells with a cell comprising a mentioned C-type lectin can be at least in part inhibited using a specific binding partner for a C-type lectin mentioned above. Thus interaction of preferably dendritic cells and said subset of NK cells and granulocytes can be interfered with. Preferably, said C-type lectin comprises DC-SIGN. As specific binding partner of said C-type lectin a mannose containing glycoconjugate such as a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a derivative or multimer thereof, can be advantageously used. The cellular interaction of DC with granulocytes or NK cells is essential in the innate immune response. In particular granulocytes are involved early ingestion of pathogens and may attract and stimulate DC to participate in pathogen recognition and presentation to T cells. In contrast NK cells are well know to be involved in killing of infected cells. NK cells can kill or activate DC that allow lysis of pathogen captured DC or maturation of pathogen captured DC to enhance T cell stimulation and immune response. The cellular interaction of granulocytes and NK cells with DC is to enhance immunity against pathogens or eliminate pathogen infected DC.

In another aspect the invention provides a method for modulating the stimulating immune effect of a dendritic cell that is activated via a Toll-like receptor signaling pathway, said method comprising contacting said dendritic cell with an isolated and/or recombinant specific C-type lectin binding molecule. Preferably, said C-type lectin comprises DC-SIGN and said specific C-type lectin binding compound comprises a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a derivative, a combination or multimer thereof and

preferably, a glycoconjugate comprising at least two mannose residues in α1,2 linkage, preferably ManLAM. In combination with Toll-like receptor signaling ManLAM binding to DC-SIGN on a dendritic cell induces the production of significant amounts of IL-10 and reduces DC-activation/maturation. The production of IL-10 and reduction of activation/maturation of DC lead to a dampening of immune responses. Thus by providing a glycoconjugate comprising at least two mannose residues in α1,2 linkage or an analogously acting glycoconjugate to a dendritic cell, this cell can be induced to dampen the immune response at least in its direct vicinity. Thus an active immune system comprising a number of dendritic cells participating in the activity can be dampened through by providing the dendritic cell with a glycoconjugate comprising at least two mannose residues in α1,2 linkage or an analogously acting glycoconjugate. This embodiment is useful in situations where an individual is suffering from or at risk of suffering from an over-active immune system. Preferably, said over-active immune system involves graft versus host disease, host versus graft disease and the various auto-immune diseases. Alternatively, an immune system dampened through the discussed mechanism can be stimulated by providing dendritic cells with a specific C-type lectin binding molecule capable of interfering with the binding of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting glycoconjugate. ManLAM, at least in amounts active locally, is tolerated by individuals considering that active amounts of ManLAM are secreted by mycobacterium infected cells in the body. Alternatively, the dampened system can be stimulated by using a ligand binding molecule of the invention, preferably a proteinaceous molecule such as a carbohydrate specific antibody preferably specific for any mannose containing glycoconjugate such as ManLAM or a glyconjugates comprising a fucose residue or a derivative or multimer thereof. Preferably, said antibody comprises a specificity for a mannose residue in α1,2 linkage with another mannose. The experimental part describes non-limiting examples of such antibodies. The ligand binding

molecule can also be a C-type lectin, preferably a soluble derivative thereof, comprising specificity for a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glyconjugates comprising a fucose residue or a derivative or multimer thereof. Another non-limiting example of such an interfering molecule is AraLAM. This embodiment is useful to combat (chronic) diseases wherein at least part of the phenotype of the disease is due to dampening of the immune system through the mentioned C-type lectin pathway. Preferred examples of such diseases are infections by a human immunodeficiency virus, a mycobacterium, a helicobacter, a *leishmania*, a *schistosoma*, a *klebsiella*, a herpes simplex virus or an ebola virus

Immature DC are highly efficient in antigen capture and processing, whereas mature DC are specialized in the naïve T cell activation necessary for cellular immune responses. Immature DC mature in response to specific 'danger' signals such as bacterial components (LPS) or inflammatory cytokines (TNFa, PGE2). In the present invention it was found that a glycoconjugate comprising a mannose, a fucose residue or a derivative, a combination or multimer thereof, does not induce DC maturation. In contrast to LPS that triggers TLR4, no up-regulated expression of the activation markers CD80, CD83, CD86 or HLA-DR was observed. However, when the maturation of immature dendritic cells was studied in the situation when the cells are also activated via a Toll-like receptor pathway, a strong inhibition of Toll-like receptor induced maturation was observed, particularly when the dendritic cells were exposed to ManLAM. Such inhibition thus dampens the response of Toll-like receptor pathway stimulated dendritic cells and thereby the capacity of an individual to cope with pathogens that are combated via activation of the Toll-like receptor pathway. This dampening of DC-activation can be at least in part prevented by at least in part inhibiting binding of particularly a glycoconjugate comprising at least two mannose residues in α1,2 linkage, but also analogously acting glycoconjugates to C-type lectins on the immature dendritic cells. The

invention thus provides a method for determining whether a compound is capable of modulating an activation state of a dendritic cell comprising providing said dendritic cell with a compound capable of specifically binding to a c-type lectin and determining whether a Toll-like receptor signaling pathway in said dendritic cell is modulated. Further provided is a method for modulating the activity of a Toll-like receptor signaling pathway in a cell, wherein said cell comprises a Toll-like receptor and a C-type lectin, said method comprising contacting said cell with an isolated and/or recombinant C-type lectin binding molecule. Preferably said C-type binding molecule comprises a glycoconjugate comprising a mannose, a fucose residue or a derivative, a combination or multimer thereof. Activity can be modulated upward by at least in part preventing simultaneous stimulation of C-type lectin with a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compound. Activity is modulated downward by simultaneously providing said a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compound. Analogously acting compounds comprise the same Toll-like receptor signaling interfering capacity as ManLAM in kind not necessarily in amount. A preferred analogously acting compound comprises a glycoconjugate comprising a fucose residue or a derivative or multimer thereof. A further level of control is possible using C-type lectin binding compounds that at least in part interfere with the binding capacity of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or an analogously acting compound. In this way activity of the Toll-like receptor pathway can be modulated upward (in the presence of the proper Toll-like receptor ligand) even in the presence of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compound. The latter feature is of importance in cases where the immune system has problems combating an infection via the Toll-like receptor pathway, particularly when said infection is accompanied with secretion of a glycoconjugate comprising at least two mannose residues in α1,2

linkage or analogously acting compounds, such as mycobacteria and in particular *M. tuberculosis* and *M. Bovis*. Thus the invention further provides a method for stimulating maturation of a dendritic cell that is contacted with a Toll-like receptor ligand and a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compound, said method comprising providing said dendritic cell with a C-type lectin binding molecule capable of blocking the binding of said glycoconjugate to said C-type lectin. C-type lectin binding molecules capable of at least in part inhibiting the binding of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compounds are for instance C-type lectin binding antibody or a functional part, derivative and/or analogue thereof, with a binding specificity that blocks or covers the ManLAM binding site on said C-type lectin. Preferable said C-type lectin comprises DC-SIGN. Thus in a preferred embodiment said antibody is a DC-SIGN specific antibody. A suitable example of such an antibody is AZN-D1, AZN-D2 or AZN-D3 or a human or humanized analogue comprising the same binding specificity in kind not necessarily in amount. In a preferred embodiment the invention provides a method for stimulating maturation of a dendritic cell that is contacted with a Toll-like receptor ligand and a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compound, said method comprising providing said dendritic cell with a ligand binding molecule of the invention, thereby at least in part preventing binding of said a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compound to the dendritic cell.

An important aspect of the invention is concerned with the use of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a derivative or multimer thereof, a ligand binding molecule of the invention and/or a C-type lectin binding molecule of the invention for the preparation of a medicament. Such

medicaments may be used for the treatment of an immune system associated disease or the treatment of an acquired disease. Preferably, said acquired disease comprises an infection with human immunodeficiency virus, mycobacteria, a fungus, a *helicobacter*, a *leishmania*, a *schistosoma*, a *klebsiella*, a herpes simplex virus or an ebola virus.

Considering the natural role of C-type lectins on antigen presenting cells it is within the scope of the present invention to stimulate immune responses in an individual by providing antigen through a C-type lectin receptor on the antigen presenting cell. This can of course be achieved when the antigen comprises a a glycoconjugate comprising at least two mannose residues in α1,2 linkage or glycoconjugate comprising a fucose residue or derivative or multimer thereof. In this embodiment it is preferred that simultaneous activation of the Toll-like receptor signaling pathway is at least in part prevented. Provided is thus a glycoconjugate comprising an antigen and a glycoconjugate comprising at least two mannose residues in α1,2 linkage and/or a fucose residue or a derivative or multimer thereof for use for the preparation of a vaccine. The vaccine may be preventive or curative. The antigen may be derived from any source as long as it is capable of being presented through major histocompatibility complex I, complex II or C1b. In a preferred embodiment said antigen comprises a tumor antigen. The presence of the mentioned carbohydrates on tumor cells facilitates antigen capture by DC to enhance antigen presentation and as a result immune activation. On the other hand, the immune response against tumor antigen lacking the mentioned carbohydrates can stimulated significantly by providing the antigen with one or more of the mentioned carbohydrates thereby stimulating DC uptake and thus the immune response against the antigen. This aspect is useful in the preparation of vaccines.

In another embodiment the invention provides the use of ligand binding molecule and/or a c-type lectin binding molecule of the invention for the preparation of a vaccine. A dampening of the immune system due to the effect of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compounds is reduced by providing a glycoconjugate or antibody capable of at least in part inhibiting the binding of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compound to their C-type lectin receptor. The antibody preferably comprises SMLDN1.1, SMFG4.1, 6H3 or SMLDN1.1. Thus antigen present in said vaccine or provided separately is more effective in stimulating or boosting an immune response in the presence of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compound. This is particularly important in patients suffering from a mycobacterial infection, such as but not limited to M. tuberculosis or *M. bovis*. Preferably, said vaccine is used to stimulate an antigen specific immune response in said individual. In a preferred embodiment the medicament or vaccine is used for the treatment of an individual suffering from a cancer, an autoimmune disease or a transplantation related disease.

As used herein the term "antibody" refers to antibodies derived from humans or other animals. The antibody is preferably produced outside the body. The antibody can also be generated or selected using artificial systems such as phage display selection. An antibody having no natural counterpart is therefore well within the scope of the present invention. Antibodies as used herein also include fragments thereof capable of binding to the same target, such as FAB fragments or even smaller parts. The antigen binding part of an antibody of the invention may also be grafted onto another type of molecule to provide that molecule with a binding specificity as provided for in the invention. Modification of the antibody to include human or humanized versions thereof with the same binding specificity in kind not necessarily in

amount are of course also in the scope of the invention. Also included are single chain fragments and variants thereof.

As used herein the term proteinaceous molecule refers to a peptide, a poly-peptide, protein and the like with or without modifications. Such modifications may be synthetic and/or provided for by a biological system. The latter including for instance post-translation modification such as glycosylation.

Where the invention is described for use in humans, the invention is also functional in other animals for instance farm-animals and pets. These medical and vaccine uses and methods of treatment are therefore also part of the invention.

Brief description of the drawings

Figure 1. Le^x – neoglycoconjugates bind with high affinity to DC-SIGN. a. Schematic diagram of the covalent structures of the glycan chains of synthetic glycoconjugates used in the DC-SIGN binding assay. b and c. Glycoconjugates were coated and binding of recombinant DC-SIGN-Fc was measured after incubation with peroxidase-labeled goat-anti-human Fc. Anti-DC-SIGN monoclonal antibody AZN-D1 were used to block binding. c. Titration of the glycoconjugates revealed that DC-SIGN binds with high affinity to Le^x , Le^y, Le^a, Le^b, or LDNF –glycoconjugates and α1,3, α1,6 mannotriose, sulfo-Lea, whereas it has a lower binding affinity for α-mannose and α-L-fucose, and sialyl-Le^x.

Figure 2. Cellular DC-SIGN displays a binding specificity similar to that of soluble DC-SIGN-Fc. Binding of DC-SIGN expressing K562 transfectants and immature monocyte derived DC to glycoconjugate-coated fluorescent beads

was measured by FACscan analysis. Binding was inhibited by anti-DC-SIGN monoclonal antibody AZN-D2. One representative experiments out of three is shown. SD is less than 2 %. Inlay: Picture of Le^x-coupled fluorescent beads that bind to DC-SIGN expressing DC.

Figure 3. DC-SIGN binds four novel pathogens

a. Pathogens that consist out of Le^x –rich Helicobacter pylori and Schistosoma mansoni, and mannose-capped lipoarabinanomannan (manLAM) of Mycobacterium tuberculosis and mannose-capped lipophosphoglycan of Leishmania mexicana, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Staphylococcus aureus were coated and binding of recombinant DC-SIGN-Fc was measured as with peroxidase-labeled goat-anti-human Fc.

b. DC-SIGN can bind LPS of Klebsiella Len1 that contains mannose cap, but does not bind the LPS of Klebsiella Len 111 that lack a mannose cap indicating that strains that contain the DC-SIGN binding carbohydrates interact with DC-SIGN on Dendritic cells or DC-SIGN transfectants.

Figure 4. DC strongly interact with H. pylori LPS through DC-SIGN. Biotinylated LPS (strains 11637 and M1019) was coated on streptavidin beads and the adhesion to DC was determined with the fluorescent bead adhesion assay. Specificity was determined by using antibodies against DC-SIGN and the mannose receptor. Moreover, adhesion was determined in the presence of mannan and EGTA.

Figure 5. DC-SIGN rapidly internalizes Le^x glycans to lysosomal compartments. Synthetic biotinylated Le^x –PAA were added to DC-SIGN-expressing K562 cells or immature monocyte derived DC for 30 min incubation at 37°C. Cells were stained with anti-CD107a (LAMP-1, green) as a lysosomal

marker and avidine-Alexa 594 for localization of Le^x after permeabilization of the cells. Co-localization of LAMP-1 and Le^x results in a yellow staining.

Figure 6. DC-SIGN specifically binds ManLAM, a cell-wall component of M. tuberculosis.

a) DC-SIGN interacts with several mycobacteria strains. DC-SIGN-Fc binding to mycobacteria ($5*10^5$ bacteria) was determined by a Fc-specific ELISA. Specificity was determined by measuring binding in the presence of blocking DC-SIGN-specific antibodies (AZN-D1 or AZN-D3) and mannan. EGTA was used to determine the calcium-dependency of the DC-SIGN-Fc-mediated binding. ICAM-3-Fc binding to mycobacteria was also measured to exclude non-specific binding by the Fc domain. Standard deviation < 0.02 OD450. One representative experiment out of three is shown. Binding of Mycobacterium Smegmatis that lacks the ManLAM structure does not interact with DC-SIGN (data not shown).

b) The schematic structure of ManLAM. Lipoarabinomannan (LAM) of M. tuberculosis consists of a glycosylphosphatidyl anchor (GPI) , an mannose-rich oligosaccharide core and a branched arabinose polymer that ends in mannose-caps (n=0-3). AraLAM has a similar structure but does not contain the mannose-cap (ref).

c) The mannosylated-lipoarabinomannan ManLAM, in contrast to the non-mannosylated AraLAM, is specifically bound by DC-SIGN. The anti-DC-SIGN antibody AZN-D1 was used to determine specificity. The DC-SIGN-Fc binding assay was performed as described in Figure 1a. Standard deviation < 0.02 OD450. One representative experiment out of three is shown.

d) DC-SIGN does not interact with AraLAM. The DC-SIGN-Fc binding assay was performed as described in Figure 1a. One representative experiment out of three is shown.

Figure 7. Cellular DC-SIGN binds strongly to both viable mycobacteria and the mycobacterial component ManLAM through its primary binding site.

a) K562-DC-SIGN transfectants express high levels of DC-SIGN but lack expression of the other reported ManLAM receptors MR, CD11b and CD11c. Transfectants were generated as previously described **Hier een referentie naar 12 ?**. Open histograms represent the isotype controls, and filled histograms indicate the specific antibody staining.

b) DC-SIGN, expressed by K562 transfectants, binds strongly to intact *M. bovis* BCG and the mycobacterial component ManLAM but not to AraLAM. The adhesion of cells to the LAM glycans was determined using the fluorescent bead adhesion assay. Binding to viable mycobacteria was determined by measuring the binding of K562 transfectants to FITC-conjugated mycobacteria (MOI 20) using flow cytometry. Specificity was determined by measuring binding in the presence of blocking antibodies against DC-SIGN. Standard deviation for the fluorescent bead adhesion assay and the mycobacteria binding assay was < 5% and <2%, respectively. One representative experiment out of three is shown.

c) The Val351 amino acid residue is not essential for the interaction of DC-SIGN with *M. bovis* BCG and ManLAM, similar to HIV-1 gp120, whereas it is essential for ICAM-3 binding. Binding to the V351G DC-SIGN mutant expressed by K562 cells was measured as described in Figure 2. Specificity was determined by measuring binding in the presence of blocking antibodies against DC-SIGN, mannan or EGTA. Standard deviation <5% (fluorescent bead adhesion assay) and <2% (mycobacteria binding assay). One representative experiment out of three is shown.

d)Binding of DC-SIGN to differential components of ManLAM. Neoglycoproteins Ara-Man, Ara-Man2, Ara-Man3 or Ara6 were coated on fluorescent beads and added to DC-SIGN expressing cells. DC-SIGN preferably binds Man2 and Man3 as demonstrated with the strong binding that can be blocked by the addition of anti-DC-SIGN antibodies (AZN-D1).

Figure 8. DC-SIGN is an important receptor for both ManLAM and mycobacteria on DC.

a) Immature DC express high levels of DC-SIGN and the other reported LAM receptors MR, CD11b and CD11c. Open histograms represent isotype control and filled histograms indicate specific antibody staining.

b) Immature DC bind strongly to ManLAM via DC-SIGN. Binding was determined using the fluorescent bead adhesion assay. Specificity was determined by measuring binding in the presence of mannan, EGTA or blocking antibodies against DC-SIGN (AZN-D2), MR (Clone 19), CD11b (bear-1) or CD11c (SHCL3). Standard deviation <5%. One representative experiment out of three is shown.

c) DC-SIGN mediates capture of *M. bovis* BCG by immature DC. Binding was determined by flow cytometry using FITC-conjugated mycobacteria. Specificity was determined by measuring binding in the presence of antibodies against DC-SIGN (AZN-D1, AZN-D2 and AZN-D3), MR (Clone 19), CD11b (bear-1) and CD11c (SHCL3). Binding was also measured in the presence of the C-type lectin inhibitors mannan and EGTA, whereas a known MR ligand, mannose-BSA, was used to determine the contribution of the MR receptor. Standard deviation <2%. One representative experiment out of three is shown.

d) DC-SIGN mediates capture and internalization of *M. bovis* BCG by K562 cells. K562 transfectants were incubated with FITC-conjugated *M. bovis* BCG (MOI 20). Cells were washed, and surface FITC was quenched by exposure to trypan blue. Phagocytosis was determined by comparing the FITC labeling before and after quenching using flow cytometry. Surface bound bacteria are represented by open bars, internalized by closed bars. Standard deviation <4%. One representative experiment out of three is shown.

e) Immature DC rapidly phagocytose mycobacteria through DC-SIGN. The internalization was determined as described in Figure 3b. Surface bound

bacteria are represented by open bars, internalized by closed bars. Standard deviation <5%. One representative experiment out of three is shown.

Figure 9. DC-SIGN mediates internalization of captured mycobacteria and ManLAM.

a) *M. bovis* BCG and ManLAM are internalized by DC-SIGN on immature DC and targeted to the lysosomes. The fate of captured mycobacteria was followed by analyzing immature DC pulsed with FITC-conjugated *M. bovis* BCG (MOI 20) for 2 hours using immuno-fluorescence microscopy (magnification 200x). ManLAM was followed by incubating DC with ManLAM (10 mg/ml) for 1 hour. DC-SIGN, ManLAM and CD207a/Lamp-1 were stained with AZN-D1, F30.5 and H4A3, respectively. One representative experiment out of three is shown.

b) ManLAM induces down-regulation of DC-SIGN, but not of MR, CD11b and CD11c. Immature DC were incubated with 15 mg/ml of ManLAM or AraLAM for 18 hours, and then DC-SIGN expression was determined by flow cytometry. One representative experiment out of three is shown.

Figure 10. Mycobacteria induce IL-10 production by DC through ManLAM and direct infection.

a) ManLAM induces IL-10 production of LPS-matured DC. Immature DC were incubated with 15 mg/ml of either ManLAM or AraLAM in the presence of LPS (10 ng/ml). The specificity was determined in the presence of blocking antibodies against DC-SIGN (AZN-D2; 20 μg/ml). Supernatants were harvested after 18 hours and the IL-10 production was measured by ELISA. Values are the means ± standard deviations of triplicate determinations. One representative experiment out of three is shown.

b) *M. bovis* BCG infection of immature DC induces IL-10 production. Immature DC were infected with *M. bovis* BCG (MOI 4), and the experiment was performed as described in Figure 5a. Values are the means ± standard

deviations of triplicate determinations. One representative experiment out of three is shown.

Figure 11. ManLAM inhibits LPS-induced DC activation through DC-SIGN binding.

a) ManLAM does not induce activation of immature DC. Immature DC were incubated with ManLAM, AraLAM or LPS for 18 hours, and activation was determined by measuring the expression of CD80, CD86, CD83 and HLA-DR. Dotted lines represent isotype controls, the thin lines indicate expression levels of immature DC, and the thick line represents immature DC that have been treated with either LPS (10 ng/ml), ManLAM (15 μg/ml) or AraLAM (15 μg/ml). One representative experiment out of three is shown.

b) LPS-induced activation of DC is blocked by ManLAM. Immature DC were co-cultured with LPS alone, or together with either ManLAM or AraLAM for 18 hours. Dotted lines represent isotype controls. Thick lines, and the mean fluorescence values in the histograms, represent the expression levels after treatment with LPS alone, or in combination with either ManLAM or AraLAM. Thin lines indicate the presence of antibodies against DC-SIGN throughout the incubation. One representative experiment out of three is shown.

Figure 12. *M. bovis* BCG induces maturation and ManLAM inhibits the induced DC activation through DC-SIGN binding.

a) *M. bovis* BCG induces maturation of immature DC. Immature DC were incubated with LPS or viable *M. bovis* BCG (MOI 4, 20 and 100) for 18 hours, and activation was determined by measuring the expression of CD80, CD86, CD83 and HLA-DR. The mean fluorescence intensity is depicted. One representative experiment out of three is shown.

b) *M. bovis* BCG-induced activation of DC is blocked by ManLAM. Immature DC were infected with *M. bovis* BCG (MOI 4). Cells were pre-incubated with

15 μg/ml of either ManLAM or AraLAM and the expression of the markers was measured after 18 hours as described in Figure 7a. Specificity was determined by pre-incubating cells with blocking antibodies against DC-SIGN (AZN-D2; 20 μg/ml). One representative experiment out of three is shown.

Figure 13. Structures of S. mansoni SEA carbohydrate antigens, monoclonal antibodies (Mabs) recognizing them, and reactivity of these Mabs with SEA. The reactivity of the MAbs was measured by ELISA, using SEA coated at a concentration of 1 μg/ml. The antibodies, incubated at a concentration of 1 μg/ml all showed a strong reaction with SEA.

Figure 14. Interaction of SEA with immature human DC.
a) Immature human DC were cultured from monocytes in the presence of GM-CSF and IL-4. The DC express next to high levels of DC-SIGN, the MR, CD83, CD86, CD80 and HLA-DR as determined by FACSscan analysis
b) DC-SIGN, expressed by immature DC, shows a similar binding to SEA as to HIV-1 gp120, a previously defined ligand of DC-SIGN. The adhesion to both SEA and HIV-1 gp120 was determined using the fluorescent bead adhesion assay. Mannan and anti-DC-SIGN MAb AZN-D1 (20 μg/ml), but not anti-MR MAb clone 19, block adhesion of SEA to the DC. One representative experiment out of three is shown, using anti-LDN antibodies to couple SEA to the fluorescent beads. Similar results are obtained using anti-LDN-DF antibodies (not shown).

Figure 15: Binding of DC-SIGN to S. mansoni SEA.
DC-SIGN binds as strongly to SEA as to HIV-1 gp120, as was determined by an ELISA based assay using soluble DC-SIGN-Fc. SEA were coated at a concentration of 5 μg/ml, and HIV-1 gp120 at a concentration of 1 μg/ml.

Specificity was determined by measuring binding in the presence of the anti-DC-SIGN blocking antibody AZN-D1 (20 μg/ml), or EDTA (5 mM).

Figure 16. DC-SIGN strongly binds SEA
Binding of soluble DC-SIGN-Fc to different concentrations SEA was measured by an anti-IgG-Fc ELISA.

Figure 17. DC-SIGN binds a subfraction of SEA
SEA were separated by SDS-PAGE gelelectrophoresis on a 12.5% polyacrylamide gel.
a) polyacrylamide gel with SEA (15 μg) silver-stained to detect all proteins
b) Western blot of polyacrylamide gel after SDS-PAGE of SEA (2 μg), using soluble DC-SIGN-Fc to detect DC-SIGN binding glycoproteins.

Figure 18. DC-SIGN binds to fucosylated SEA and the α1,3-fucosylated trisaccharide Le^x
a) SEA were defucosylated by mild acid treatment or α3,4-fucosidase. Binding of soluble DC-SIGN-Fc to defucosylated SEA (coated at 1 μg/ml) was measured by an anti-IgG-Fc ELISA. The degree of defucosylation, and the integrity of non-fucosylated glycans after the treatments was established using different anti-glycan MAbs, and goat-anti-mouse IgM-peroxidase for detection.
b) Competitive inhibition of the binding of soluble DC-SIGNFc to SEA and HIVgp120 by anti-glycan mAbs in ELISA. Coated antigens were preincubated with anti-glycan mAbs before adding DC-SIGNFc. Binding of DC-SIGN Fc was measured by an anti-IgGFc ELISA. Antibodies usedpreferably SMLDN1.1 or SMFG4.1 (anti-Le^x) or SMLDN1.1 (anti-LDNF) or anti-DC-SIGN (AZN-D1, D2, AZN-D3),
c) DC-SIGN stongly binds to neoglycoproteins carrying α1,3-fucosylated oligosaccharides Le^x and LDN-F but poorly to neoglycoconjugates carrying a single a-linked fucose or to LDN-DF. Neoglycoproteins were coated at a

concentration of 5 μg/ml. No binding was observed to neoglycoproteins carrying Galb1,4GlcNAc or GalNAcb1,4GlcNAc (LDN) (not shown).

Figure 19. Binding of SEA and Le^x to mutant DC-SIGN and L-SIGN

a) Amino acid sequence alignment of part of the CRDs of DC-SIGN (AAK20997) with that of the highly homologous C-type lectin L-SIGN (AAK20998). The position of the E324A, E347Q and V351G mutations in DC-SIGN are indicated by an arrow.

b) Binding of SEA and Le^x-PAA coated beads to K562 transfectants expressing wild-type DC-SIGN and the E324A, E347Q and V351G DC-SIGN mutants, was measured using the fluorescent bead adhesion assay. To detect SEA, fluorescent beads coupled to the LDN-DF MAb were used. One representative experiment out of two is shown.

c) SEA blocks binding of DC-SIGN, expressed by human immature DC, to ICAM-3 and HIV-1-gp120. In contrast, RNAseB does not block this binding. The adhesion of both ICAM-3 and HIV-1 gp120 to DC was determined using the fluorescent bead adhesion assay. Inhibitors were present at a concentration of 20 μg/ml.

d) DC-SIGN, expressed by K562 transfectants, binds strongly to both SEA and HIV-1 gp120, whereas L-SIGN, expressed by K562 transfectants only recognizes HIV-1 gp120. The adhesion was determined using the fluorescent beads adhesion assay. To detect SEA, both anti-LDN and anti-LDN-DF coated fluorescent beads were used with similar results. One representative experiment out of two is shown, using anti-LDN-DF fluorescent beads.

e) K562 transfectants efficiently express DC-SIGN, L-SIGN and the different mutant DC-SIGNs, as determined by FACSscan analysis

Figure 20. Granulocytes strongly interact with DC-SIGN-coated beads, partly through Le^x-containing ligands.

A. Both mannose as well as Lewis-X epitopes are highly expressed on glycans expressed by granulocytes

B. DC-SIGN binds strongly to freshly isolated granulocytes. Freshly isolated granulocytes are incubated with DC-SIGN-Fc-coated fluorescent beads. The adhesion is determined by the fluorescent bead adhesion assay. Specificity is determined by measuring the adhesion in the presence of mannan, EGTA and antibodies against DC-SIGN (AZN-D1,D2 or D3), b2 integrins (NKI-L19) and Le^x (6H3)

Figure 21. DC-SIGN-Fc has a high affinity for granulocytes.
The interaction of DC-SIGN with granulocytes is further investigated by titration of DC-SIGN-Fc and determining the binding by measuring the bound DC-SIGN-Fc using FITC-conjugated Goat-anti-human Fc antibodies. The interaction is blocked by antibodies against DC-SIGN (AZN-D1), and mannan and EGTA (A) and by anti-Le^x antibodies (3H3, B).

Figure 22. A 66kDa protein (CD66) is the high affinity ligand of DC-SIGN on granulocytes.
A. Granulocytes are surface labelled with biotine. The cell lysate is incubated over night at 4°C with Protein A beads coated with DC-SIGN-Fc or control antibodies against ICAM-2, ICAM-3 and LFA-1. The beads are washed and the immuno-precipitated product is analysed on SDS-PAGE and visualised by autoradiograph. In particular a protein size 66kD is immuno-precipitated. B. Granulocytes highly express the Le^x containing CD66acd and CD66d antigen, do not express the DC-SIGN ligand ICAM-2 and express ICAM-3. C. Immuno-precipitation of CD66acd demonstrates strong binding activity for DC-SIGNFc. Anti-CD66acd mAbs coated on an ELISA plate were incubated with a lysate of granulocytes. After washing off non-specific proteins DC-SIGNFc was incubated to demonstrate high affinity ligands. DC-SIGNFc does not recognize ICAM-3 from granulocytes demonstrating that on granulocytes not ICAM-3

but CD66acd is the high affinity ligand for DC-SIGN. Binding of CD66acd to DC-SIGNFc is completely inhibited by removal of cations by EDTA.

Figure 23. Cellular DC-SIGN strongly binds to the granulocyte-specific protein CD66a.

293T cells are transfected with pIG-CD66a-Fc, pIG-ICAM-3-Fc and pIG (mock). Supernatants are harvested and beads are coated with the Fc chimeras (CD66a-Fc, ICAM-3-Fc and mock). Immature DC and DC-SIGN-transfected K562 cells are incubated with beads and the adhesion is determined in the presence of antibodies against DC-SIGN and β2 integrins, and mannan and EGTA.

Figure 24. NK cells specifically interact with DC-SIGN-Fc.

The binding of DC-SIGN-Fc to peripheral blood lymphocyte (PBL) subsets is determined by flow cytometry in the presence of antibodies against DC-SIGN. PBL subsets are distinguished by staining with CD3 and CD56, and adhesion is determined by triple staining with DC-SIGN-Fc.

Figure 25. NK cells specifically interact with DC-SIGN-Fc in a concentration-dependent manner. The binding of DC-SIGN-Fc to peripheral blood lymphocyte (PBL) subsets is determined by flow cytometry in the presence of antibodies against DC-SIGN. NK cells are detected by triple staining with CD3, CD56 and DC-SIGN-Fc. The CD56 dim and bright cells do not differ in ICAM-2 or ICAM-3 expression demonstrating that differential glycosylation is the result of the high affinity binding of DC-SIGNFc to CD56 dim NK cells.

Figure 26. $CD56^{dim}CD16^{+}$ NK subset specifically interacts with DC-SIGN-Fc.

The binding of DC-SIGN-Fc to NK subsets is determined by flow cytometry in the presence of antibodies against DC-SIGN. NK subsets are distinguished by

staining with CD16 and CD56, and the adhesion is determined by triple staining with DC-SIGN-Fc.

Figure 27. A 166kDa protein and ICAM-2 is recognized by DC-SIGN-Fc on NK cells.
NK cells are surface labelled with radioactive iodine. The cell lysate is incubated over night at 4°C with Protein A beads coated with DC-SIGN-Fc or control antibodies against ICAM-2, ICAM-3 and LFA-1. The beads are washed and the immuno-precipitated product is analysed on SDS-PAGE and visualised by autoradiography.

Figure 28. Maturation of DC by NK cells is mediated by LFA-1.
Immature DC are co-cultured with NK cells for 24 hours in the presence of antibodies against DC-SIGN (AZN-D1) and LFA-1 (NKI-L19). The maturation is determined by measuring the expression of CD80, CD83, CD86 and HLA-DR. with NK cells O/N at 37°C. As a control, DC are matured with LPS (10 ng/ml).

Figure 29. NK-mediated lysis of immature DC is dependent on DC-SIGN.
Chromium^{52}-labeled immature or LPS-matured DC are co-cultured with IL-2-activated or non-activated NK cells for 4 hours in the presence of antibodies against DC-SIGN (AZN-D1) and LFA-1 (NKI-L19). The lysis is determined by measuring the Cr^{52} release.

Figure 30. NK-mediated lysis of K562 transfectants is enhanced by DC-SIGN.
Chromium^{52}-labeled K562 cells are co-cultured with IL-2-activated or non-activated NK cells for 4 hours in the presence of antibodies against DC-SIGN. The lysis is determined by measuring the Cr^{52} release.

Figure 31. DC-SIGN has a high affinity for HSV-1 and HSV-2.

HSV is coated in ELISA plates and DC-SIGN-Fc binding is determined in the presence of antibodies against DC-SIGN.

Figure 32. DC-SIGN strongly binds HSV envelope glycoprotein gB.
A.DC-SIGN transfectants are incubated with HSV envelope glycoprotein-coated beads and DC-SIGN binding is determined in the presence of antibodies against DC-SIGN.
B L-SIGN strongly binds HSV envelope glycoprotein gB.
L-SIGN transfectants are incubated with HCV envelope glycoprotein-coated beads and DC-SIGN binding is determined in the presence of antibodies against DC-SIGN.

Examples

Methods.

Antibodies and proteins. The following monoclonal antibodies (Mab) were used: anti-CD107a (Lamp-1; MabH4A3, BD Pharmingen)., anti-MR (Clone 19, BD Pharmingen), CD11b (bear-1)[25], CD11c (SHCL3)[26], anti-DC-SIGN (AZN-D1, AZN-D2[14], CSRD[10], blocking CD11a (NKI-L15), anti-ICAM-1 (Rek-1), anti-ICAM-2(12A2), and anti-ICAM-3 (AZN-IC3/1, icr-2), activating CD18 (KIM185) and the PE/FITC-conjugated antibodies CD25, CD69, CD80, CD86, HLA-DR (BD Pharmingen), and CD83 and CD56 (Beckman Coulter). Biotin-conjugated CD3 and CD16 (BD Pharmingen). CLB-T4 (αCD4) was a kind gift of René van Lier. MEM-157 (αCD16) and MEM-97 (αCD20) were kindly provided by Vaclav Horejsi. ICAM-1 mAb CBR-IC-1/3, ICAM-2 mAb CBR-IC-2/1, and ICAM-3 mAb CBR-IC-3/2 were obtained through the Human Leukocyte Differentiation Antigen Workshop. Several anti-glycan monoclonal antibodies (MAbs) were used: the anti-LDN-DF MAb 114-5B1-A[16], the anti-LDN MAb SMLDN1.1[17], the anti-LDN-F MAb SMLDNF1[10] and the anti-Le^x

MAb CB10[27] , the anti-Lex mAbs SMLDN1.1 and SMFG4.1 and6H3 (anti-Lex). The mAb (CLB-gran/10) was used to stain and immunoprecipitate CD16.

The neoglycoprotein HSA-Le^x, containing approx. 20-25 oligosaccharide chains per HSA molecule, was from Isosep AB, Tullinge, Sweden. The neoglycoprotein BSA-LDN-DF (approx. 12 oligosaccharide chains per molecule BSA), and BSA-LDN-F (approx. 3-4 oligosaccharides per molecule BSA), were synthesized enzymatically as described by van Remoortere et al[16], and Nyame et al[17] , respectively. Le^x-PAA-biotin, containing Le^x multivalently coupled to biotinylated polyacrylamide was from Syntesome, Munich, Germany.

Mannan purified from *Saccheromyces cerevisiae* (50 µg/ml) and recombinant gp120 (0.50 µg/ml) were obtained from Sigma and the Aidsresource Foundation, respectively. Purified mannose capped lipoarabinomannan (manLAM) from *Mycobacterium tuberculosis*, and non-capped LAM (araLAM) from *M. smegmatis* were kindly provided by Dr. J. Belisle, Colorado State University through NIH, NIAID contract NO1 AI-75320. Soluble egg antige (SEA) was kindly provided by Dr. A. K. Nyame, Oklahoma University HSC, USA). Purified lipophosphoglycan from *Leishmania mexicana* was kindly donated by Dr. M Wiese, Bernard Nocht Inst. Tropical Medicine Hamburg, Germany. Purified lipopolysaccharide of *Helicobacter pylori* was obtained from M. Monteiro, NRC, Ottawa, Canada. A sonicate of bacterial cells of a clinical isolate of *M. tuberculosis* was donated by A. Kolk, KIT, Royal Trop. Inst, Amsterdam. Clinical isolates of *Helicobacter pylori, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus* were obtained from VUMC Hospital, Amsterdam. Synthetic glycoconjugates were obtained from Syntesome, Munich, Germany, and comprise mono- and oligosaccharides multivalently linked to a biotinylated polyacrylamide carrier, MW 40.000.

Soluble DC-SIGN-Fc adhesion assay. DC-SIGN-Fc consists of the extracellular portion of DC-SIGN (amino acid residues 64-404) fused at the C-terminus to a human IgG1-Fc fragment. DC-SIGN-Fc was produced in Chinese Hamster Ovary K1 cells by co-transfection of DC-SIGN-Sig-pIgG1 Fc (20 μg) and pEE14 (5 μg) vector. DC-SIGN-Fc concentrations in the supernatant were determined by an anti-IgG1 Fc ELISA. The DC-SIGN-Fc binding assay was performed as follows. Glycoconjugates and sonicated mycobacteria were coated onto ELISA plates at 5 μg/well; intact bacterial cells were coated at $5x10^7$/ml ; coating took place for 18 hours at room temperature, followed by blocking with 1% BSA for 30 min, at 37°C in TSM (20mM Tris-HCl pH 7.4 containing 150mM NaCl, 2 mM $CaCl_2$ and 2mM $MgCl_2$). Soluble DC-SIGN-Fc (approx. 2 μg/ml in TSM buffer) was added and the adhesion was performed for 120 min. at RT. Unbound DC-SIGN-Fc was washed away and binding was determined by an anti-IgG1 Fc ELISA using a peroxidase conjugate of goat anti-human Fc. Specificity was determined in the presence of either 20 μg/ml blocking antibodies, 50 μg/ml mannan or 5mM EGTA.

Cells. Immature DC were cultured from monocytes in the presence of IL-4 and GM-CSF (500 and 800 U/ml, respectively; Schering-Plough, Brussels, Belgium[28]. At day 7 the phenotype of the cultured DC was confirmed by flow cytometric analysis. The DC expressed high levels of MHC class I and II, αMβ2 (CD11b), αXβ2 (CD11c) and ICAM-1, moderate levels of LFA-1 and CD80, and low levels of CD14. K562 transfectants expressing wild-type DC-SIGN were generated by transfection of K562 cells with 10 μg pRc/CMV-DC-SIGN plasmid by electroporation as previously described.

Stable transfectants of K562 expressing ICAM-3 were obtained by electroporation of pCDM8-ICAM-3 and pGK-HYG. K562 cells were cultured on RPMI 10% FCS, whereas K562-ICAM-3 cells were cultured on RPMI 10% FCS : Iscove's 5% FCS 3:1 containing 0.5 mg/ml hygromycine to maintain ICAM-3 expression.

Naive NK cells were isolated from buffy coats of healthy donors. Briefly, the PBMC fraction obtained through Ficoll centrifugation was sequentially depleted for $CD14^+$ cells, and $CD3^+$, $CD4^+$, and $CD20^+$ cells using MACS sorting. $CD14^+$ cells were depleted by CD14 microbeads (Miltenyi Biotec) on an LS column (Miltenyi Biotec), and $CD3^+$, $CD4^+$, and $CD20^+$ cells were labeled by the mAbs T3B (αCD3), CLB-T4 (αCD4), MEM97 (αCD20), and thereafter depleted by goat-α-mouse microbeads (Miltenyi Biotec) on an LD column (Miltenyi Biotec). Thus obtained NK cells were routinely tested for the NK cell markers CD16 (75% to 90% expression), CD56 (80 to 95% expression), the non-lineage markers CD3, CD4, CD14 and CD20 (all less than 1% expression), and the early activation marker CD69 (15% expression on non-activated NK cells and 75% expression on 1-day IL-2-activated NK cells). $CD56^{dim}$ and $CD56^{bright}$ NK cell populations were isolated from MACS obtained NK cells by FACS sorting on low and high CD56 expression, respectively. $CD56^{dim}$ and $CD56^{bright}$ NK cell populations were over 95% pure as assessed by flow cytometry staining for CD16 and CD56.

DC activation. Immature DC ($2x10^6$ cells/ml) were cultured for 24 hours in the presence of IL-4 (500 U/ml, Schering-Plough, Brussels, Belgium), GM-CSF (800 U/m; Schering-Plough, Brussels, Belgium) and either LPS (10 ng/ml) or LAM glycolipids (15 μg/ml). The effect of LAM on LPS-induced activation was determined by pre-incubating immature DC (300.000 cells) with AZN-D2 (40 μg/ml) for 30 minutes, and subsequently with LPS in the presence of LAM (15 μg/ml) for 18 hours. LAM glycolipids were obtained from J. Belisle (Colorado State University and the NIH (contract NO1 AI-75320)) and contained <5 ng/mg endotoxin. Activation was determined by cell-surface expression of MHC class II (HLA-DR) and the co-stimulatory molecules CD80, CD83 and CD86 using PE-conjugated antibodies.

Fluorescent bead adhesion assay. Carboxylate-modified TransFluorSpheres (488/645 nm, 1.0 μm; Molecular Probes, Eugene, OR) were coated with HIV-1 gp120 and ICAM-3 as described[14]. Streptavidin was covalently coupled to the beads as described and streptavidin-coated beads were incubated with biotinylated PAA-linked glycoconjugates (50 pMol; Syntesome, Munich, Germany). The fluorescent bead adhesion assay was performed as described[14]. Ligand-coated fluorescent beads (20 beads/cell) were added to the cells for 45 minutes at 37°C, washed and analyzed by flow cytometry (FACScan, Becton Dickinson, Oxnard, CA), by measuring the percentage of cells that had bound fluorescent beads. LAM-coated beads were generated by coating incubating streptavidin-coated beads were incubated with biotinylated F(ab')2 fragment goat anti-mouse IgG (6 μg/ml; Jackson Immunoresearch) followed by an overnight incubation with mouse-anti-LAM antibody (F30.5) at 4°C. The beads were washed and incubated with 250 ng/ml purified glycolipid LAM (obtained from J. Belisle, Colorado State University and the NIH (contract NO1 AI-75320)) overnight at 4°C. SEA-coated beads were generated by incubating the streptavidin-coated beads with biotinylated F(ab')2 fragment of goat anti-mouse IgG (6 μg/ml; Jackson Immunoresearch), followed by an overnight incubation at 4°C with anti-LDN MAb, or anti-LDN-DF MAb. The beads were washed and incubated with 1μg/ml SEA overnight at 4°C. Essentially, $50x10^3$ cells were pre-incubated in adhesion buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1mM CaCl2, 2 mM MgCl2, 0.5% BSA) with or without blocking MAbs (20 μg/ml) or mannan (50 μg/ml) for 10 minutes at room temperature. Ligand-coated fluorescent beads (20 beads/cell) were added to the cells and the suspension was incubated for 45 minutes at 37°C. Cells were washed and adhesion was determined using flow cytometry (FACScan, Becton Dickinson, Oxnard, CA), by measuring the percentage of cells that had bound fluorescent beads. HIV-1 gp120 fluorescent beads were prepared as described previously[19].

DC-SIGN-Fc adhesion. Cells were incubated with DC-SIGN-Fc for 30 minutes at 37°C under saturating conditions (concentration: 10 μg/ml), and subsequently with FITC-conjugated goat-α-human secondary antibodies to monitor adhesion of DC-SIGN-Fc. Before incubation with cells, DC-SIGN-Fc was preincubated for 10 min at RT either with medium, aDC-SIGN (AZN-D1, 50 μg/ml), mannan (50 μg/ml), or EGTA (10 mM) to determine specificity of DC-SIGN-Fc adhesion. DC-SIGN-Fc adhesion was determined by flow cytometry (FACS Calibur, Beckman Coulter).

Mycobacteria. Both the *M. bovis* BCG (Pasteur) and the *M.tuberculosis* H37Ra strains were gifts from A. Kolk (Royal Tropical Institute, Amsterdam). *M. bovis* BCG was cultured *in vitro* using Middelbrook 7H9 broth supplemented with 0.05% Tween 80 and albumin-dextrose-catalase. The glycolipids ManLAM and AraLAM were obtained from J. Belisle, Colorado State University and the NIH (contract NO1 AI-75320). DC were infected with mycobacteria by co-culturing them at an appropriate multiplicity of infection (MOI) as indicated in the figure legends.

Fluorescent mycobacterial binding assay. Capture and internalization of mycobacteria by cells was evaluated using fluorescein isothiocyanate (FITC)-conjugated *M. bovis* BCG. Bacteria (10^9/ml) were labeled by incubation of 0.5 mg FITC per ml in phosphate buffered saline (pH 7.4) at room temperature for 1 hour. The FITC-pulsed bacteria were washed three times to remove unbound FITC. Capture was determined by measuring the percentage of cells that bound FITC-conjugated bacteria using flow cytometry (FACScalibur, Becton Dickinson Immunocytometry, San Jose, CA). Phagocytosis was determined using a fluorescence-quenching technique as reported previously[29]. In brief, quenching of non-internalized membrane-bound FITC-conjugated *M. bovis* BCG was achieved by treating the cells with 0.05% trypan blue for 5 minutes.

H. pylori binding was assessed by labeling the bacteria with FITC and binding to DC was investigated similarly to the bead assay.

Cytokine production. For the detection of cytokines, culture supernatants were harvested at day 1 and frozen at -80°C until analysis. The supernatants were analyzed for the presence of IL-10 and IL-12p40 by ELISA (Biosource International, CA).

Defucosylation of SEA. SEA were defucosylated by incubation of the antigens at 100°C for 1 hour in 0.1M TFA. After neutralization, the defucosylated antigen was coated to ELISA plates. Enzymatic defucosylation was performed by incubating the SEA in 50 mM sodiumphosphate pH=5.0 with α1,3/4-fucosidase (0.4 mU/µg SEA) (Calbiochem) overnight at 37°C. The degree of defucosylation of the antigens was assessed by their ability to bind MAbs specifically recognizing the fucosylated glycan epitopes LDN-DF and Le^x, whereas the integrety of other, non-fucosylated, glycan epitopes was assessed by measuring the reactivity with anti-LDN MAb.

SDS-PAGE, Western blotting and silver staining. SEA were separated by SDS-PAGE under reducing conditions on a 12,5% polyacrylamide gel, using the Mini-Protean II system (BioRad), and proteins visualized by silver-staining. For Western blotting proteins were transferred onto a nitrocellulose membrane (Schleicher and Schuell). The membrane was blocked in a solution of 5% BSA in TSM for 2 h followed by incubation in 2 µg/ml DC-SIGN-Fc in TSM buffer containing 1% BSA for 1 h. After washing, the membrane was subsequently incubated for 1 h in peroxidase conjugated goat anti-human IgG1 and reactive bands were visualized by detection with CN/DAB substrate (Pierce).

Cytotoxicity. The standard 4-hour ^{51}Cr release assay was used to assess NK cell-mediated cytotoxicity. Briefly, $1*10^6$ target cells were labeled by 100 µCi

^{51}Cr for 1 hour at 37°C, extensively washed to remove free ^{51}Cr, resuspended at 2500 cells/well (iDC and mDC) or 1000 cells/well (K562 and K562-DC-SIGN), and incubated with NK cells for 4 hours at 37°C at the indicated ratios and under the indicated conditions. After 4 hours scintillation liquid (PerkinElmer) was added to supernatants, and ^{51}Cr release was determined on a micro-b counter (PerkinElmer).

NK cell-mediated DC maturation. Resting and activated NK cells were obtained by overnight incubation on medium and IL-2 (1000 U/ml), respectively. Thereafter, resting and activated NK cells were incubated overnight with immature DC in a 96 wells U-bottom plate (Costar) on RPMI 10% FCS. NK cells were preincubated for 10 min. at RT with medium or blocking αLFA-1 mAbs (NKI-L15, 50 μg/ml), whereas DC were preincubated with medium or blocking αDC-SIGN mAbs (AZN-D2, 50 μg/ml). As a positive control DC were incubated overnight in the presence of LPS (2 μg/ml). Maturation of DC was assessed by flow cytometry (FACS Scan, Beckman Coulter) for the maturation markers CD80, CD83, CD86, and HLA-DR by PE-conjugated or FITC-conjugated mAbs.

DC-induced NK cell activation. Resting NK cells were incubated for 2 days with immature or mature DC (obtained by LPS maturation) in a 96 wells U-bottom plate (Costar) on RPMI 10% FCS. LFA-1-dependent DC-induced NK cell activation was determined by a 10 min. preincubation of NK cells with αLFA-1 mAbs (NKI-L15, 50 μg/ml), whereas dependency on DC-SIGN was assessed by a 10 min. preincubation of DC with anti-DC-SIGN mAbs (AZN-D1 and AZN-D2, 50 μg/ml). As a positive control NK cells were incubated for 2 days with IL-2 (1000 U/ml). NK cell activation was assessed by flow cytometry (FACS Calibur, Beckman Coulter) for the early activation marker CD69 by FITC-conjugated mAbs.

Immunoprecipitation. NK cells were surface iodinated with 1 mCi ^{125}I, or biotinylated and subsequently lysed in lysisbuffer (1% Triton-X -100, 10 mM TEA, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF, 20 μg/ml trypsin inhibitor, 20 μg/ml leupeptin, and 20 μg/ml aprotinin). DC-SIGN ligands, ICAM-2, ICAM-3, and LFA-1 were immunoprecipitated from prot A-precleared NK cell lysate by prot A beads covalently linked to DC-SIGN-Fc, αICAM-2 (12A2), αICAM-3 (AZN-IC-3/1), and αLFA-1 (NKI-L15). Immunoprecipitates were reduced in sample buffer (containing 4% SDS and 5% b-mercapto-ethanol), heated for 5 min at 95°C, and run on a 5-15% gradient polyacrylamide gel (SDS-PAGE).
Alternatively, specific anti-bodies against CD66acd or ICAM-3 or LFA-1 were coated in an ELISA plate, subsequently lysates were added and incubated for 1 hr at 4C to retract the specific proteins. DC-SIGNFc was incubated for binding activity to the specific proteins and stained with Goat anti human Fc PO and developed.

Experimental setup.

Dendritic cells (DC) are instrumental in handling pathogens for processing and presentation to T cells, thus eliciting an appropriate immune response. C-type lectins expressed by DC function as pathogen-recognition receptors; yet their specificity for carbohydrate structures on pathogens is not fully understood. Here we analyzed the carbohydrate specificity of DC-SIGN/CD209, the recently documented HIV-1 receptor on DC. Our studies show that DC-SIGN binds with high affinity to both synthetic mannose- and fucose- containing glycoconjugates. These carbohydrate structures are abundantly expressed by pathogens as demonstrated by the affinity of DC-SIGN for natural surface glycans of the human pathogens *Mycobacterium tuberculosis, Helicobacter pylori, Leishmania mexicana* and *Schistosoma*

mansoni. Strikingly, these pathogens target DC-SIGN to infect DC but also to modulate the immune responses mediated by DC.

This analysis expands our knowledge on the carbohydrate and pathogen-specificity of DC-SIGN, and identifies this lectin to be central in pathogen-DC interactions.

Example 1: Novel Carbohydrate specificity for DC-SIGN

For *in vitro* binding studies we generated a chimeric protein of DC-SIGN with a human IgG1 Fc tag, which we used to screen in an ELISA format for reactivity with a panel of synthetic glycoconjugates containing mannose or fucose residues and their derivatives in multimeric form.

As reported earlier, DC-SIGN-Fc binds to purified yeast-derived mannan and the high mannose containing HIV-1 gp120, but also to less complex mannose-containing glycoconjugates i.e. mannose and α1->3, α1->6mannotriose (Table I, Figure 1). Strikingly, DC-SIGN binds to Lewis blood group antigens (Le^x, Le^y, Le^a, Le^b), glycan comprising at least one terminal fucose α1,3 or α1,4-linked to N-acetylglucosamine (Le^x, Le^y Le^a, Le^b, LDNF), or α-1,2 to Galactose (Le^y, Le^b) (Table I, Figure1). Sialylation of Le^x (yielding sialyl-Le^x, a L-, E- and P-selectin ligand) completely abrogates the recognition by DC-SIGN, indicating that DC-SIGN has a carbohydrate specificity that is distinct from that of the selectins that mediate leukocyte rolling. Sulfation reduced the binding affinity of DC-SIGN for Le^x, as well as Le^a (Figure 1c). To compare in more detail the affinity of DC-SIGN binding to Le^x and α1>3, α1>6-mannotriose, titration studies were performed with the different DC-SIGN binding glycoconjugates (Figure 1c). Strikingly, DC-SIGN binds with much higher affinity to the fucose-containing carbohydrate Le^x than to mannotriose. The binding activity of DC-SIGN-Fc to these glycan structures was specific, since anti-DC-SIGN antibodies blocked the interaction (Figure 1c).

To determine whether DC-SIGN-Fc exhibits a similar carbohydrate recognition profile as cell-surface expressed DC-SIGN, both DC-SIGN transfectants and monocyte-derived DC were studied for carbohydrate binding activity using a fluorescent beads adhesion assay with different glycoconjugates (α1->3, α1->6mannotriose, Le^x and sulfo-Le^a) (Figure 2). Indeed, DC-SIGN expressed by K562 transfectants bound similarly to the glycoconjugates as DC-SIGN-Fc and the binding was completely inhibited by anti-DC-SIGN antibodies (Figure 2). Even though DC express many other C-type lectins on their cell surface, our data demonstrate that the glycoconjugates containing Le^x and α1->3, α1->6mannotriose are preferentially bound by DC-SIGN. The interaction is specific since anti-DC-SIGN antibodies almost completely inhibited the binding activity. This illustrates that DC-SIGN is the major receptor on DC for these carbohydrate structures. Binding of sulfo-Le^a to DC could only be partially blocked by anti-DC-SIGN antibodies indicating that other C-type lectins on DC compete with DC-SIGN for binding of sulfo-Le^a . Our data show that DC-SIGN recognizes a wider range of glycan structures, including Lewis blood group antigens, than hitherto realized. Thus, DC-SIGN may be an important receptor for recognition of novel biologically relevant targets expressed by the host, or alternatively by human pathogens.

Example 2. Novel carbohydrate specificity (Le^x and high mannose) identifies novel pathogens (*Helicobacter pylori, Schistosoma mansoni, Mycobacterium tuberculosis, Leishmania mexicana*) that interact with DC-SIGN

We subsequently investigated the binding of DC-SIGN to human pathogens that express mannose- or fucose-containing glycans. The gram-negative *Helicobacter pylori* bacterium, which induces peptic ulcers and gastric carcinoma[20], and the worm parasite (the causal agent of schistosomiasis) both express Le^x [21]. In *H. pylori*, Le^x is present on surface-located

lipopolysaccharide, while in *S. mansoni* Le^x is expressed by all stages of the parasite, including soluble egg antigen (SEA)[21]. Binding of DC-SIGN-Fc to Le^x-positive *H. pylori* lysate and to extract of *S. mansoni* was strong and was completely inhibited by anti-DC-SIGN antibodies (Figure 3); When analysisng two different LPS forms from Klebsiella one containing mannose cap and one without (Len1 and Len111 respectively) (Figure 3B). DC-SIGN expressed by DC also bound to purified LPS of *H. pylori* (Figure 4). Binding of DC to both *H. pylori* cells and purified LPS could be completely blocked with anti-DC-SIGN Mab (Figure 4). Also glycans present in *Mycobacterium tuberculosis*, the causative agent of tuberculosis, are bound by DC-SIGN (Figure 3). The mannose-capped surface glycan, lipoarabinomannan of *Mycobacterium tuberculosis* probably contains the recognition site for DC-SIGN. This is further supported by the fact that DC-SIGN also bound to the mannose-capped surface lipophosphoglycan (LPG) expressed by an unicellular parasite that causes leishmaniasis (Figure 3). Binding of DC-SIGN to *Leishmania* was reported very recently[30] but we demonstrate here that LPG is the structure on *Leishmania* that is recognized by DC-SIGN (Figure 3). No binding of DC-SIGN to three clinically relevant Gram-negative bacterial human pathogens (*Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa*) was observed nor to Gram-positive *Staphylococcus aureus*. However other clinical isolates of Klebsiella that contain a mannose capped LPS bind DC-SIGN. These findings indicate that binding of DC-SIGN to pathogens is selective, and that the carbohydrate specificity of DC-SIGN governs a broader pathogen recognition than only viruses such as HIV-1 and Ebola virus[12,22].

Example 3 DC-SIGN interacts with *M. tuberculosis* through ManLAM glycolipids

Whole mycobacteria such as the *M. tuberculosis* H37Ra strain and *M. bovis bacillus* Calmette-Guérin (BCG) were coated and the interaction of

DC-SIGN with these pathogens was analyzed using the DC-SIGN-Fc binding assay. *M. bovis* BCG is a tuberculosis strain that is almost nonpathogenic yet retains the immunological properties of tuberculosis. DC-SIGN-Fc interacted specifically with both *M. tuberculosis* H37Ra and *M. bovis* BCG, since the interaction was inhibited with blocking DC-SIGN-specific antibodies (Figure 6a). *M. smegmatis*, an avirulent strain that does not contain a Mannose cap, did not bind DC-SIGN (data not shown).

Moreover, an irrelevant Fc chimera, ICAM-3-Fc, did not interact with the mycobacteria (Figure 6a). The interaction is mediated by the C-type lectin domain of DC-SIGN, since binding to both *M. tuberculosis* and *M. bovis* BCG was inhibited by EGTA, mannan and the DC-SIGN-specific antibody AZN-D1 that recognizes the lectin domain[23] (Figure 6a).

We next investigated the binding of DC-SIGN to purified mycobacterial lipoarabinomannan (LAM), since DC-SIGN has a high affinity for mannose-containing carbohydrates and LAM is the major mannose-containing component of the mycobacterial cell-wall[31]. LAM comprises a mannose-rich polysaccharide-core, containing highly branched arabinofuranosyl side chains, and a GPI anchor (Figure 6b). LAM isolated from *M. tuberculosis* contains mannose-residues consisting exclusively of mono-, di- and trimers of α-D-mannoses directly linked to the arabinofuranosyl-termini and is called ManLAM, whereas LAM isolated from the fast growing *M. smegmatis* is not mannose-capped and is called AraLAM[31] (Figure 6b). Strikingly, purified ManLAM was efficiently bound by DC-SIGN, in contrast to AraLAM (Figure 6c), demonstrating that DC-SIGN specifically interacts with the mono-, di- and trimers of α-D-mannoses of ManLAM. Even at high concentrations, DC-SIGN did not bind AraLAM, demonstrating a high specificity for ManLAM and its mannose-cap (Figure 6d). The interaction of DC-SIGN to ManLAM is specific, since the binding was inhibited by antibodies against DC-SIGN, whereas an irrelevant Fc chimera did not interact with ManLAM (Figure 6c). DC-SIGN interacts similarly with both ManLAM and

whole *M. bovis* BCG indicating that DC-SIGN binds mycobacteria through ManLAM (Figure 6a and c).

Example 4 Both mycobacteria and ManLAM interact with the primary binding site of DC-SIGN

We used K562 transfectants stably expressing DC-SIGN to investigate the binding of cell-surface-expressed DC-SIGN to both *M. bovis* BCG and the mycobacterial component ManLAM. These cells do not express the previously reported mycobacterial receptors Mannose Receptor (MR), CD11b and CD11c (Figure 7a). K562 transfectants express high levels of DC-SIGN (Figure 7a) and bind strongly to both *M. bovis* BCG and ManLAM, in contrast to mock transfected K562 cells (Figure 7b). The interaction is blocked by DC-SIGN-specific antibodies (Figure 7b). The interaction of DC-SIGN with both *M. bovis* BCG and ManLAM is similar to that of the other DC-SIGN ligands ICAM-3 and HIV-1 (Figure 7b). Thus, cellular DC-SIGN specifically binds to both *M. bovis* BCG and ManLAM, as was observed with recombinant DC-SIGN-Fc (Figure 6c).

The C-type lectin domain of DC-SIGN contains two calcium ions, and the amino acid residues that are in close contact with Ca^{2+} at site 2 (Glu^{347}, Asn^{349}, Glu^{354} and Asn^{365}) form the core of the ligand binding site[23]. Changing in DC-SIGN either Glu^{347} into Gln (E347Q), or Asn^{349} and Asn^{365} into Asp, resulted in complete loss of binding to whole mycobacteria and ManLAM (Figure 7b and data not shown), similarly as was shown previously for both ICAM-3 and HIV-1 gp120 (Figure 7b). The Ca^{2+} at site 1, the so-called auxiliary site, coordinates the correct positioning of the primary binding site, and loss of this Ca^{2+} by mutating Asp^{320}, Glu^{324} (E324A), Asn^{350} or Asp^{355} into Ala residues resulted in complete loss of both *M. bovis* BCG and ManLAM binding (Figure 7b and results not shown).

Recently, we demonstrated that the binding site of DC-SIGN for its cellular ligand ICAM-3 is distinct from that of HIV-1 gp120, since a specific mutation in DC-SIGN (V351G) abrogated ICAM-3, but not HIV-1 gp120 binding (Figure 7c). Strikingly, the DC-SIGN V351G mutant also interacts with *M. bovis* BCG as well as ManLAM (Figure 7c), demonstrating that both HIV-1 and mycobacteria bind similarly to DC-SIGN at a distinct site from the cellular ligand ICAM-3. The similar binding of both *M. bovis* BCG and ManLAM to DC-SIGN mutants further supports our findings that DC-SIGN specifically interacts with ManLAM on whole mycobacteria. Binding of fluorescent beads coated with distinct neoglycoproteins of ManLAM that consist of Arabinose, Arabinose-α1,5 mannose, Arabinose- α1,5Man- α1,2 Man, Ara- α1,5Man- α1,2Man- α1,2Man or Ara6 demonstrate that DC-SIGN particularly recognizes in ManLam a di-mannose (Man α1,2 component) or mannosetriose (Man α1,2 component) (Figure 7D).

Exampe 5 DC-SIGN is a major receptor for mycobacteria on DC

Immature DC express, besides high levels of DC-SIGN, high levels of the receptors MR, CD11b and CD11c (Figure 8a), which have previously been reported to mediate binding of mycobacteria by macrophages. We used blocking antibodies against these receptors to evaluate their contributions to ManLAM binding by DC. Immature DC bind strongly to ManLAM, but not to AraLAM, and the interaction was inhibited by the DC-SIGN-specific antibody, but strikingly not by any of the antibodies against MR, CD11b or CD11c (Figure 8b). Both EGTA and the C-type lectin-specific inhibitor mannan block ManLAM binding by DC to a similar extent as the DC-SIGN-specific antibodies, demonstrating that DC-SIGN is the primary ManLAM-binding C-type lectin on immature DC that binds ManLAM (Figure 8b).

The major contribution of DC-SIGN to the interaction of immature DC to ManLAM prompted us to investigate whether DC-SIGN could be

important in the interaction of immature DC to whole mycobacteria. Immature DC strongly interacted with *M. bovis* BCG (Figure 8c). Strikingly, DC-SIGN is a major receptor for *M. bovis* BCG, since the antibodies against DC-SIGN strongly inhibited the infection of immature DC with *M. bovis* BCG (Figure 8c). Antibodies against MR, CD11b and CD11c did not inhibit the infection, whereas the C-type lectin inhibitor mannan blocked the infection to a similar level as the DC-SIGN antibodies (Figure 8c). Moreover, the MR-ligand mannose-BSA did not inhibit the interaction of DC with *M. bovis* BCG (Figure 8c) demonstrating that the C-type lectin domains of MR are not involved in *M. bovis* BCG infection of DC. Both the anti-MR antibody and mannose-BSA are inhibitors of MR-function since they block binding of another MR-ligand dextran to DC (results not shown). These results demonstrate that DC-SIGN is the primary C-type lectin on DC that functions as a receptor for *M. bovis* BCG. Other non-lectin receptors may participate in the interaction since the infection was not completely inhibited by antibodies against DC-SIGN (Figure 8c).

Example 6 DC-SIGN facilitates capture and internalization of *M. bovis* BCG by immature DC through binding of the mycobacterial cell-wall component ManLAM.

Next we investigated whether DC-SIGN mediates internalization of *M. bovis* BCG using trypan blue to quench surface FITC-conjugated mycobacteria. Mock transfected K562 cells did not phagocytose *M. bovis* BCG, whereas 50% of the K562 transfectant, expressing DC-SIGN, had internalized *M. bovis* BCG within 45 minutes (Figure 8d). Both mannan and antibodies against DC-SIGN blocked the internalization of *M. bovis* BCG (Figure 8d).

Immature DC are highly phagocytosing cells and indeed within 45 minutes more than 90% of the dendritic cells that bound *M. bovis* BCG, had internalized the mycobacteria (Figure 8e). Similar as was observed for the

binding of *M. bovis* BCG by DC (Figure 8c), phagocytosis of *M. bovis* BCG is partly blocked by antibodies against DC-SIGN whereas both anti-MR antibodies and the MR-ligand mannose-BSA did not inhibit the observed phagocytosis (Figure 8e). These results demonstrate that DC-SIGN facilitates capture and internalization of *M. bovis* BCG by immature DC through binding of the mycobacterial cell-wall component ManLAM.

Example 7. Mycobacteria and ManLAM are internalized by DC-SIGN and targeted to lysosomes

Recently, we have demonstrated that DC-SIGN can function as an antigen receptor that internalizes antigens and targets them to lysosomal compartments for presentation on MHC Class II[10] . Therefore, the fate of the captured *M. bovis* BCG by immature DC was followed by immuno-fluorescence analyses. Immature DC were incubated with FITC-conjugated *M. bovis* BCG for 2 hours and both DC-SIGN and Lamp-1 were stained (Figure 9a). The observed co-localization of DC-SIGN with FITC-conjugated *M. bovis* BCG further supports a role for DC-SIGN in the capture and internalization of mycobacteria (Figure 9a). Phagocytosed mycobacteria are targeted to the lysosomes since the internalized FITC-conjugated mycobacteria co-localize with Lamp-1 staining (Figure 9a). Similarly, ManLAM was also captured and internalized by DC-SIGN on immature DC since ManLAM staining co-localized with DC-SIGN (Figure 9a) whereas AraLAM was not internalized by DC (results not shown). Internalized ManLAM co-localized with the lysosomal marker LAMP-1/CD107a in immature DC (Figure 9a) indicating that internalized ManLAM is targeted to lysosomes. Thus, both whole mycobacteria and the cell-wall component ManLAM are similarly internalized by immature DC through DC-SIGN, supporting the results that DC-SIGN interacts with ManLAM on mycobacteria.

Recently, we demonstrated that binding of soluble ligands or antibodies to DC-SIGN triggers internalization of the DC-SIGN-ligand complex to the late endosomes/lysosomes, and results in down-regulation of DC-SIGN from the surface[10]. Therefore, we investigated whether DC-SIGN is internalized after ManLAM binding by measuring the cell-surface expression of DC-SIGN after ManLAM binding using a specific antibody against DC-SIGN. Indeed, binding of ManLAM, but not AraLAM, to DC-SIGN results in down-regulation of DC-SIGN (Figure 9b), demonstrating that DC-SIGN on DC binds ManLAM and mediates the internalization of ManLAM to $CD107a^+$ lysosomes. Moreover, other mycobacteria-receptors such as MR, CD11b and CD11c were not downregulated (Figure 9b). Thus, ManLAM binding to DC-SIGN triggers internalization of the DC-SIGN/ManLAM complex, and might enable antigen processing of ManLAM by DC.

Example 8. ManLAM changes the cytokine production by DC through DC-SIGN

ManLAM is present not only a mycobacterial cell-wall component but is also secreted from phagosomes following macrophage ingestion of M. tuberculosis[31]. Potentially, mycobacteria within infected macrophages can influence bystander immune cells and modulate the immune response through secretion of ManLAM. The cytokine IL-10 is a potent immunosuppressive factor induced in macrophages by some intracellular bacteria to dampen down host immune responses and promote their survival[32]. We investigated the influence of ManLAM binding to DC-SIGN in IL-10 production by DC. ManLAM alone did not induce IL-10 production by immature DC (Figure 10a). Strikingly, ManLAM, but not AraLAM, strongly induced IL-10 production by DC, when they received simultaneously an activation signal, such as LPS (Figure 10a). This IL-10 induction was completely inhibited by DC-SIGN-specific antibodies to the level of LPS-activated DC alone (Figure 10a). The

findings that only ManLAM could induce IL-10 production, which could be blocked by DC-SIGN-specific antibodies indicates that the IL-10 induction is specific for the ManLAM/DC-SIGN interaction. Antibodies against DC-SIGN alone did not induce IL-10 production by LPS-activated DC (Figure 10a), demonstrating that ligation of DC-SIGN alone is not sufficient for IL-10 induction. Thus, the binding of ManLAM to DC-SIGN triggers intracellular signals that induce IL-10 production by DC, indicating that mycobacteria target DC-SIGN to suppress the immune response and promote their survival in the host. Both immature and LPS-activated DC, alone or in combination with ManLAM, produced very low amounts of IL-12p70 (<5 pg/ml). Infection of immature DC with *M. bovis* BCG induced a strong production of IL-10 that was not inhibited by antibodies against DC-SIGN (Figure 10b). No differences were observed in the presence of ManLAM (Figure 10b). These results suggest that mycobacteria induce IL-10 production by direct infection as well as by secreting ManLAM.

Example 9. ManLAM inhibits TLR4-mediated DC activation through DC-SIGN

Immature DC are highly efficient in antigen capture and processing, whereas mature DC are specialized in the naïve T cell activation necessary for cellular immune responses. Immature DC mature in response to specific 'danger' signals such as bacterial components (LPS) or inflammatory cytokines (TNFa, PGE2). We investigated the effect of ManLAM on the maturation of DC. Neither ManLAM nor AraLAM induced DC maturation, since both ManLAM and AraLAM, in contrast to LPS that triggers TLR4, did not up-regulate expression of the activation markers CD80, CD83, CD86 or HLA-DR (Figure 11a).

Acute mycobacterial infections represent sites of inflammation that attract and induce DC maturation through the presence of maturation components. Therefore, we investigated the effect of ManLAM and AraLAM in combination with the stimulatory bacterial LPS. Toll-like receptor-4 (TLR4) interaction with LPS generates intracellular signaling, most notably via the transcription factor NFkB, that results in DC activation/maturation. Indeed, DC efficiently mature in the presence of LPS alone (Figure 11b). Strikingly, this LPS-induced activation is inhibited in the presence of ManLAM, since the expression levels of the activation markers CD80, CD83 and CD86 were considerably lower than those of LPS-activated DC (Figure 11b). The observed inhibition of DC activation/maturation is specific for ManLAM, since AraLAM did not inhibit DC activation (Figure 11b). This is further supported by the ability of antibodies against DC-SIGN, that inhibit ManLAM binding, to completely restore LPS-induced maturation in the presence of ManLAM (Figure 11b). These results indicate that ManLAM binding to DC-SIGN generates intracellular signals that interfere with the TLR4-mediated activation of DC. Moreover, this process is specific for the ManLAM-DC-SIGN interaction and DC-SIGN ligation alone is not sufficient since antibodies against DC-SIGN did not block LPS-induced DC activation/maturation (Figure 11b).

Example 10. *M. bovis* BCG-induced DC maturation is inhibited by ManLAM.

Both *M. tuberculosis* and *M. bovis* BCG are able to induce DC maturation through their cell-wall components via TLR2- and TLR4-mediated signaling[33-36]. Indeed, *M. bovis* BCG infection of immature DC results in DC maturation, as demonstrated by the increased expression of MHC class II and the co-stimulatory molecules CD80, CD83 and CD86 after *M. bovis* BCG infection (Figure 12a). Next, we investigated whether ManLAM binding to DC-SIGN prevented *M. bovis* BCG-induced DC maturation, since immature DC

attracted to sites of mycobacterial infection will encounter both secreted ManLAM and intact mycobacteria. Strikingly, the *M. bovis* BCG-induced DC maturation is inhibited strongly by ManLAM (Figure 12b). The expression of MHC class II, CD80, CD83 and CD86 on *M. bovis* BCG-infected DC in the presence of ManLAM was considerably lower than on *M. bovis* BCG-infected DC (Figure 12b). Moreover, the maturation was mostly restored when DC were pre-incubated with the blocking DC-SIGN-specific antibody (Figure 12b), demonstrating that the ManLAM interaction with DC-SIGN prevents DC maturation by *M. bovis* BCG. Moreover, AraLAM did not block the DC maturation by *M. bovis* BCG (Figure 11c), since the co-stimulatory molecules, CD80, CD83 and CD86, are expressed at similar levels on both infected DC and AraLAM-treated infected DC. This indicates that the DC-SIGN-ManLAM interaction blocks the maturation of DC induced by LPS as well as *M. bovis* BCG.

Example 11. *S. mansoni* soluble egg antigens (SEA) bind to human immature dendritic cells (DC) through interaction with DC-SIGN.

Because DC are central in directing Th1-Th2 responses, we searched for a cell-surface receptor expressed on DC that interacts with *S. mansoni* SEA. To detect binding of SEA to human immature DC, a fluorescent bead adhesion assay was developed. Fluorescent beads were pre-coated with monoclonal antibodies (MAbs) to SEA glycan antigens and then used to capture SEA. The conjugated beads were allowed to interact with DC. SEA is a mixture of glycoproteins, containing many immunogenic glycan antigens (3, 36). Major glycan antigens present in SEA, and the recognition of these antigens by anti-glycan MAbs, are depicted in Figure 13. To capture SEA on the fluorescent beads, we used MAbs against GalNAcß1,4GlcNAc (LDN) and GalNAcß1,4(Fucα1,2Fucα1,3)GlcNAc (LDN-DF) (Figure 13), both epitopes that occur on many glycoconjugates within SEA and which are absent on DC.

Strong binding of SEA to DC was observed with SEA coated fluorescent beads that were coupled via anti-LDN MAb as well as via anti-LDN-DF MAb (Figure 14b). Binding of SEA to DC was comparable in strength to the binding of HIV-1 gp120 to DC. Because the C-type lectins DC-SIGN and the MR, that are expressed by immature DC (Figure 14a), are potential receptors for the recognition of glycan antigens, we investigated whether antibodies directed against the C-type lectin CRD of these molecules, or mannan hapten could inhibit binding of SEA to the DC. The binding of SEA to DC was strongly blocked both by the anti-DC-SIGN MAb AZN-D1, that binds to the CRD of DC-SIGN and by mannan, but not by the anti-MR MAb clone 19 (Figure 14b). These data indicate that DC-SIGN is not only a pathogen receptor on DC for HIV-1, but also for Schistosome SEA.

Example 12. A sub-fraction of SEA contains high affinity ligands for DC-SIGN.

To further analyze the binding properties of DC-SIGN to *S. mansoni* SEA, we investigated binding of soluble chimeric DC-SIGN-Fc to SEA. In an ELISA based assay DC-SIGN-Fc showed efficient binding to wells coated with SEA (Figure 15a). Binding of DC-SIGN-Fc to SEA is mediated by the CRD of DC-SIGN, since the interaction was completely inhibited by the anti-DC-SIGN antibody AZN-D1, or EGTA that removes Ca^{2+} ions that are essential for carbohydrate binding. Binding of DC-SIGN-Fc to SEA is likely to be high affinity, since binding was observed at very low coating concentrations of SEA (Figure 16). Because SEA contains many different glycoproteins, we investigated whether a subset of glycoproteins within SEA interact with DC-SIGN. SEA glycoproteins were separated by SDS-PAGE and analyzed by Western blotting with DC-SIGN-Fc and anti-glycan antibodies reactive with Le^x and LDN-DF, respectively. Among the proteins present in SEA a major protein of approximately 70-80 kD, and two minor high-molecular proteins showed interaction with soluble DC-SIGN-Fc (Figure 17). Remarkably, some

glycoproteins of similar apparent molecular weight as those reacting with DC-SIGN-Fc bound to MAbs specific for Le^x and LDN-DF glycans.

Example 13. DC-SIGN binds to α3-fucosylated glycans.

Many glycoproteins of SEA are heavily fucosylated (3, 36). Because it has been reported that DC-SIGN may exhibit binding to both mannose and fucose, and Western blotting of SEA suggested that Le^x and/or LDN-DF glycans are present on SEA proteins with similar apparent MW as the SEA proteins binding to DC-SIGN, we explored whether defucosylation of SEA affects its recognition by DC-SIGN-Fc. Treatment of SEA with the Xanthomonas α1,3/1,4-fucosidase resulted in 50% decreased reactivity of anti-Le^x MAb to SEA, whereas no loss in reactivity was observed, as expected, with anti-LDN-DF Mab. The LDN-DF epitope contains terminal α2-linked fucose. The results thus indicate that the that the glycosidase treatment specifically removed part of the α3-fucose moieties present on SEA. The loss in a3/4-fucose residues in SEA upon treatment with the Xanthomonas α1,3/α1,4-fucosidase also resulted in a 25% loss of binding of DC-SIGN-Fc (results not shown). These data suggest that α1,3/α1,4-linked fucose residues may be important for binding of soluble DC-SIGN-Fc to SEA.

Next we investigated whether one or more of the fucosylated SEA antigens Le^x, LDNF or LDN-DF (see Figure 13) may function as ligand(s) for DC-SIGN on SEA. We analyzed the potential of DC-SIGN-Fc to bind to neoglycoproteins containing these glycan antigens in ELISA. The results show that DC-SIGN-Fc strongly interacts with the neoglycoprotein HSA-Le^x and to a lesser extent with BSA-LDNF, which both carry a terminal α3-fucose. By contrast, DC-SIGN-Fc does not bind BSA-LDN-DF in which the α3-fucose is capped with an α2-fucose (Figure 18). The binding is fucose-dependent, since no binding was observed to neoglycoproteins carrying Galβ1,4GlcNAc (LN) or GalNAcβ1,4GlcNAc (LDN). The binding to Le^x and LDNF by DC-SIGN is

mediated through its CRD, since binding was inhibited by anti-DC-SIGN and EDTA. In conclusion, these results indicate that DC-SIGN strongly recognizes the α3-fucosylated trisaccharide Le^x and most likely interacts with α3-fucosylated glycans on SEA such as Le^x and/or LDNF.

Example 14. The amino acid residue Val^{351} within DC-SIGN is crucial for binding of DC-SIGN to both SEA and Le^x

The C-type lectin domain of DC-SIGN binds two Ca^{2+} ions and those amino acid residues in close contact with Ca^{2+} at site 2 (Glu^{347}, Asn^{349}, Glu^{354} and Asn^{365}) or with Ca^{2+} at site 1 (Asp^{320}, Glu^{324}, Asn^{350} and Asp^{355}) are essential for ligand binding[37]. Because the results above indicated that the α3-fucosylated trisaccharide Le^x on SEA may function as a ligand for DC-SIGN, we investigated the binding properties of these antigens to K562 cell transfectants expressing mutated forms of DC-SIGN. Mutation of either E^{324} to Ala, or E^{347} to Gln in DC-SIGN (Figure 19a) resulted in complete loss of interaction with both SEA and Le^x, indicating that binding to these antigens is mediated through the primary ligand-binding site and is Ca^{2+} dependent (Figure 19b). This is similar to results reported for binding of DC-SIGN to HIV-1 gp120 [37].

It has recently been demonstrated that a specific mutation in the CRD of DC-SIGN ($V^{351}G$) allows binding of HIV-1 gp120, but abrogates binding to ICAM-3, a T-cell ligand that has been shown previously to interact with DC-SIGN. Our results indicate that the DC-SIGN $V^{351}G$ mutant does not bind to either SEA or Le^x, whereas binding to HIV-1 was still observed, indicating that Val^{351} is essential for binding to both SEA and Le^x, but not to HIV-1 (Figure 19b). To determine whether the SEA binding site overlaps with the ICAM-3 or HIV-1 binding sites on DC-SIGN, we explored whether SEA inhibits the interaction of DC-SIGN to ICAM-3 and HIV-1 gp120 in a DC-binding assay. The results demonstrate that SEA can inhibit the interaction

between DC and ICAM-3 as effectively as the anti-DC-SIGN MAb, whereas binding of DC-SIGN to HIVgp120 was only partially inhibited by SEA (Figure 19c). These results demonstrate that the SEA binding site on DC-SIGN may resemble the ICAM-3 binding site and may partly overlap the binding site for HIV-1 gp120.

Example 15. L-SIGN does not interact with DC-SIGN binding SEA or Lewis X

L-SIGN, an adhesion receptor that resembles DC-SIGN by recognizing ICAM-2, ICAM-3 and HIV-gp120, contains a CRD that is nearly identical to that of DC-SIGN[38], and both receptors recognize high-mannose-type N-glycans. Our results above demonstrated a novel binding activity of DC-SIGN to fucosylated glycans and showed that Val^{351} in DC-SIGN is essential for this binding activity. At the corresponding position in the CRD of L-SIGN a Ser is present instead of Val (Figure 19a), raising the question whether L-SIGN can recognize SEA and Le^x. Binding of L-SIGN to these antigens was investigated by an adhesion assay where fluorescent beads containing SEA and Le^x-PAA, respectively, were incubated with transfected K562 cells expressing recombinant DC-SIGN and L-SIGN (Figure 19e). K562 cells expressing L-SIGN (K-L-SIGN) did not interact with SEA nor with Le^x-PAA, but showed interaction with HIV-1 gp120. K562 cells expressing DC-SIGN (K-DC-SIGN) bound to SEA and Le^x, as expected (Figure 19b). These data show that the two highly related receptors DC-SIGN and L-SIGN have distinct carbohydrate binding specificity and pathogen-recognition features.

Example 16. Novel carbohydrate specificity predicts and confirms novel cellular counter-structures for DC-SIGN; DC-SIGN recognizes Le^x carbohydrates on CD66a present on granulocytes to mediate DC-granulocyte interactions

DC-SIGN is known to interact with ICAM-2 and ICAM-3, however the glycan ligands on these molecules have not yet been identified. The blood group antigen Le^x (CD15) is expressed by gastric mucosal epithelial cells, and by polymorphonuclear leukocytes (granulocytes) (Figure 20a); indeed DC-SIGN-Fc-coated beads bind strongly to granulocytes (Figure 20b). Moreover, DC-SIGN-Fc also binds strongly to granulocytes (Figure 21). The binding is specific for DC-SIGN since it can be inhibited by anti-DC-SIGN antibodies, mannan and depletion of calcium by EGTA but also by an anti-Le^x antiobidy indicating that the cellular interaction is Le^x dependent (Figure 20 and 21). Moreover, an antibody against Le^x specifically reduces the observed adhesion of DC-SIGN to CD66a demonstrating that Le^x carbohydrates indeed participate in the interaction (Figure 20). Other glycan structures such as Le^y and Le^b can also be involved since the observed block is not complete. This demonstrates that granulocytes express a cell-surface glycoprotein that expresses a glycan structure that is recognized by DC-SIGN.

Moreover, we predict that based on the carbohydrate recognition pattern of DC-SIGN (Figure 1), DC-SIGN may also mediate binding of DC to tumor cells, since Le^y expression is increased on many carcinomas including ovary, pancreas, prostate, breast, colon and non-small cell lung cancers[24], while sulfo-Le^a is present on certain tumors that express mucins. Thus, these results indicate that recognition of distinct carbohydrate structures by DC-SIGN may allow DC mediated cell adhesion to T cells, endothelial cells, PMNs as well as to tumor cells.

Immuno-precipitation studies revealed that DC-SIGN-Fc does not bind the cellular DC-SIGN counter-structures ICAM-2 or ICAM-3 from granulocytes but binds to a novel glycoprotein with a weight of 66kD protein that is only present on granulocytes (Figure 22a). The fact that DC-SIGN binds Le^x carbohydrates and the fact that granulocytes express high levels of CD66a, a Le^x bearing cell-surface receptor with a molecular weight of 66kD (Figure 22b and c), prompted us to examine binding of CD66a-Fc binding to

DC-SIGN expressing transfectants and immature DC that express DC-SIGN. Strong binding of CD66a to DC-SIGN expressed by both DC and DC-SIGN transfectants was observed (Figure 23c), similar as was observed for ICAM-3 binding (Figure 23b). Un-coated beads did not interact with these cells (Figure 23a). The interaction of CD66a with DC-SIGN seemed stronger than the previously described ICAM-3-DC-SIGN interaction. These data indicate that CD66a is a novel cellular counter-structure of DC-SIGN and may play an important role in mediating DC-granulocyte cellular interactions that play a role in innate immune responses.

Example 17. DC-SIGN binds strongly to a subset of cells in peripheral blood.

To determine whether DC-SIGN preferentially interacts with naïve T cells expressing high levels of ICAM-3, as was previously postulated, we started to investigate DC-SIGN binding to total PBL population of cells. Strikingly, DC-SIGN-Fc interacted with a $CD3^-$ cell population that were $CD56^+$ and belonged to NK cell subset (Figure 24). Moreover, the interaction was concentration dependent (Figure 25) and could be blocked with antibodies against DC-SIGN (Figure 24 and 25). The $CD56^+$ NK cells in blood can be divided into two cell populations: $CD56^{dim}CD16^+$ and $CD56^{high}CD16^-$ NK cells (Figure 26). DC-SIGN-Fc specifically bound to the $CD56^{dim}CD16^+$ NK population and the interaction could be inhibited by anti-DC-SIGN antibodies (Figure 26). ICAM-1-Fc did not interact with the $CD56^{dim}CD16^+$ NK population, demonstrating that the interaction of DC-SIGN-Fc is specific and not mediated by the Fc tag. Both NK cell populations were able to specifically interact with ICAM-1-Fc after activation of their β2 integrin LFA-1 (Figure 26). Thus, these results indicate that DC interact with the $CD56^{dim}CD16^+$ NK cells through DC-SIGN. Strikingly, both NK populations have similar levels of ICAM-2 and ICAM-3, indicating that DC-SIGN may bind another novel ligands on the $CD56^{dim}CD16^+$ NK population. Indeed, immuno-precipitation

reveals that DC-SIGN-Fc binds, besides ICAM-2, also a molecule with molecular weight of 166 kDa (Figure 27). This ligand could be CD166 or CD16, which is heavily glycosylated and only expressed on NK cells.

Example 18. DC-SIGN mediates DC-NK cell interaction and is involved in the lysis of immature DC by NK cells.

To investigate whether the interaction of DC-SIGN with NK cells is relevant in NK mediated immune response we invested both the lysis of DC by NK cells and the maturation of DC by NK cells; two processes that have been recently identified to play an important role in innate immunity.

DC-SIGN did not inhibit DC maturation by NK cells (Figure 28) but was strongly involved in the NK-mediated immature DC lysis (Figure 29), since antibodies against DC-SIGN inhibited the NK-mediated lysis of DC. These data indicate that DC-SIGN regulates the interaction of immature DC with NK cell by binding a 166 kDa protein on $CD56^{dim}CD16^{+}$ NK cells. This DC-SIGN-NK cell interaction also strongly enhances NK-mediated lysis of cell-lines transfected with DC-SIGN (Figure 30), whereas antibodies against DC-SIGN inhibit the lysis. These results indicate that DC-SIGN may be important in the interaction of DC with NK cells and that inhibition of this interaction prevents NK-mediated lysis.

Example 19. DC-SIGN interacts with Herpes simplex virus type 1 and 2

Previous results demonstrated that DC-SIGN specifically interacts with HIV-1[39] and recently it was published that DC-SIGN binds to Ebola virus[40]. Therefore, we investigated whether DC-SIGN and its homologue L-SIGN can interact with other viruses, that contain glycosylated envelope proteins. Strikingly, DC-SIGN-Fc binds strongly to Herpes simplex virus (HSV)-1 and –2, and this interaction is specically inhibited by antibodies

against DC-SIGN (Figure 31). Further analysis demonstrates that DC-SIGN interact with the HSV glycoprotein gB (Figure 32) since DC-SIGN expressed by DC and transfectants binds strongly to gB-coated beads. This interaction is blocked with anti-DC-SIGN antibodies (Figure 32). DC-SIGN is specifically expressed by DC and L-SIGN is expressed by Liver sinusoidal endothelial cells (LSEC) and some macrophage populations. These data indicate that both DC-SIGN and L-SIGN could be involved in the interaction of HSV with DC and LSEC, respectively. Indeed, DC strongly bind to HSV-1 gB and the interactions are mediated by DC-SIGN, since antibodies against DC-SIGN block this interaction (Figure 35). Thus, the interaction of DC with HSV is mediated by DC-SIGN and this C-type lectin could be important in the infection of DC by these viruses. Moreover, these data could suggest that these viruses target DC-SIGN on DC not only to infect these DC but also to evade the immune response by modulating DC function as we demonstrated for mycobacteria and HIV-1.

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Claims

1. A method for at least in part inhibiting binding of a C-type lectin or a carbohydrate-binding part thereof to a ligand of said C-type lectin, comprising providing a binding molecule capable of specifically blocking binding of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or glycoconjugate comprising a fucose residue or a derivative or multimer thereof, to said C-type lectin.

2. A method according to claim 1, wherein said binding molecule is specific for a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a derivative or multimer.

3. A method according to claim 1 or claim 2, further comprising a cell comprising said C-type lectin.

4. A method according to claim 3, wherein said cell comprises an antigen presenting cell.

5. A method according to claim 4, wherein said cell comprises a dendritic cell or a macrophage.

6. A method according to any one of claims 1-5, wherein said C-type lectin comprises DC-SIGN, L-SIGN and/or DC-SIGNR, or a DC-SIGN homologue.

7. A method according to any one of claims 1-6, wherein said fucose is linked to an aniomer and wherein said linkage allows binding of said glycoconjugate to said C-type lectin.

8. A method according to any one of claims 1-7, wherein said glycoconjugate comprising a fucose residue comprises Lewis bloodgroup antigen, Le^x, Le^y, Le^a, Le^b or LDNF or a C-type lectin binding part, derivative and/or analogue thereof.

9. A method according to any one of claims 1-8, wherein said ligand comprises a (tumor) antigen, a pathogen and/or a cell associated receptor.

10. A method according to claim 9, wherein said cell associated receptor comprises ICAM-2, ICAM-3, CD166 or CD66 or a functional part, derivative and/or analogue thereof.

11. A method according to claim 9, wherein said pathogen comprises a virus, a (myco)bacterium, a fungus or a parasite.

12. A method according to claim 11, wherein said pathogen comprises a human immunodeficiency virus, a helicobacter, a leishmania, a schistosoma, a klebsiella, a herpes simplex virus or an ebola virus.

13. Use of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a derivative or multimer thereof for at least in part inhibiting the binding of a ligand to a C-type lectin or a lectin-binding part thereof.

14. Use of a specific binding partner of a C-type lectin for at least in part inhibiting binding of a cell comprising said C-type lectin to an NK-cell, a granulocyte, a T cell or a tumor cell.

15. Use of carbohydrate binding molecule specific for a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a fucose residue or a derivative or multimer thereof binding molecule, for at least in part inhibiting the binding of said glycojungate to a C-type lectin.

16. A use according to claim 15, wherein said carbohydrate binding molecule comprises an antibody or a soluble derivative of said C-type lectin.

17. A use according to claim 15 or claim 16, wherein said C-type lectin is present on a cell.

18. A use according to claim 14 or claim 17, wherein said cell is a dendritic cell or a macrophage.

19. A use according to any one of claims 13-19, wherein said binding partner of said C-type lectin comprises a glycoconjugate comprising at least

two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a derivative or multimer thereof.

20. A method for modulating the activity of a Toll-like receptor signaling pathway in a cell, wherein said cell comprises a Toll-like receptor and a C-type lectin, said method comprising providing a binding molecule capable of specifically blocking binding of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or glycoconjugate comprising a fucose residue or a derivative or multimer thereof, to said C-type lectin.

21. A method according to claim 21, wherein said binding molecule is specific for a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a derivative or multimer.

22. A method according to claim 20, wherein said binding molecule is a C-type lectin binding molecule comprising a glycoconjugate comprising a mannose, a fucose residue or a derivative, a combination or multimer thereof.

23. A method according to claim 22, wherein said C-type binding molecule comprises a glycoconjugate comprising a mannose or a derivative, or multimer thereof.

24. A method according to claim 23, wherein said C-type binding molecule comprises a glycoconjugate comprising at least two mannose residues in α1,2 linkage or analogously acting compound.

25. A method according to any one of claims 20-24, wherein said cell is contacted with a ligand for said Toll-like receptor.

26. A method for stimulating maturation of a dendritic cell that is contacted with a Toll-like receptor ligand and a glycoconjugate comprising a mannose, a fucose residue or a derivative, a combination or multimer thereof, said method comprising providing said dendritic cell with a binding molecule capable of blocking the binding of said glycoconjugate to said C-type lectin.

27. A method according to claim 26, wherein said dendritic cell is provided with a binding molecule specific for a glycoconjugate comprising at

least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue or a derivative or multimer.

28. A method according to claim 26 or claim 27, wherein said binding molecule comprises an antibody or a functional part, derivative and/or analogue thereof.

29. A method according to claim 26 wherein said antibody is a C-type lectin specific antibody.

30. Use of a glycoconjugate comprising mannose or a fucose residue or a derivative or multimer thereof for the preparation of a medicament.

31. Use of a binding molecule specific for a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glyconjugates comprising a fucose residue or derivative or multimer thereof for the preparation of a medicament.

32. A use according to claim 31 or claim 32, for the preparation of a medicament for the treatment of an immune system associated disease.

33. A use according to claim 31 or claim 32, for the preparation of a medicament for the treatment of an acquired disease.

34. A use according to claim 33, for the treatment of an individual suffering from an infection with human immunodeficiency virus, mycobacteria, a *helicobacter*, a *leishmania*, a *schistosoma*, a *klebsiella*, a herpes simplex virus, or an ebola virus.

35. Use of a glycoconjugate comprising and antigen and a fucose residue or a derivative or multimer thereof, for the preparation of a vaccine.

36. A use according to claim 35, for stimulating an antigen specific immune response in said individual.

37. A use according to any one of claims 30-36, for the treatment of an individual suffering from a cancer, an autoimmune disease or a transplantation related disease.

38. A method for determining whether a compound is capable of modulating an activation state of a dendritic cell comprising providing said

dendritic cell with a compound capable of specifically binding to a c-type lectin and determining whether a Toll-like receptor signaling pathway in said dendritic cell is modulated.

39. Use of a glycoconjugate comprising a mannose or a fucose for separating a DC-SIGN positive cell from a DC-SIGN negative cell.

40. Use of a DC-SIGN or a carbohydrate binding part, derivative and or analogue thereof for purifying a molecule comprising a glycoconjugate comprising at least two mannose residues in α1,2 linkage or glycoconjugate comprising a fucose residue or a derivative or multimer thereof.

41. A water soluble proteinaceous molecule comprising a carbohydrate binding part of a C-type lectin.

42. A water soluble proteinaceous molecule according to claim 41, comprising a carbohydrate binding part of DC-SIGN.

43. A water soluble proteinaceous molecule according to claim 42, further comprising a part of an immunoglobulin.

44. An antibody comprising a binding specificity for a carbohydrate binding part of DC-SIGN or a functional part, derivative and/or analogue thereof.

45. An antibody comprising a binding specificity for a glycoconjugate comprising a fucose residue or a glycoconjugate comprising a mannose residue.

46. An antibody according to claim 45, comprising no binding specificity for said glycoconjugate in the absence of said fucose or mannose residue.

47. An antibody according to claim 45 or claim 46, wherein said antibody comprises a binding specificity for at least two mannose residues in α1,2 linkage.

48. An antibody according to any one of claims 44-47, wherein said antibody is herein identified as SMLDN1.1, SMFG4.1, 6H3, AZN-D1, AZN-D2 or AZN-D3 or a functional part, derivative and/or analogue thereof.

49. A human or humanized antibody comprising an antigen binding part of an antibody according to claim 48.

50. Use of an antibody according to any one of claims 44-49 or a water-soluble proteinaceous molecule comprising a carbohydrate binding part of a C-type lectin for the preparation of a medicament.

51. Use according to claim 50, for the treatment of an infection with a pathogen, preferably, of human immunodeficiency virus, a mycobacterium, a fungus, a helicobacter, a leishmania, a schistosoma, a klebsiella, a herpes simplex virus or an ebola virus.

52. Use of an antibody according to any one of claims 44-49 or a water-soluble proteinaceous molecule comprising a carbohydrate binding part of a C-type lectin for at least in part preventing binding of a glycoconjugate comprising at least two mannose residues in α1,2 linkage or a glycoconjugate comprising a fucose residue to the C-type lectin DC-SIGN.

Title: C-type lectin binding molecules, identification and uses thereof.

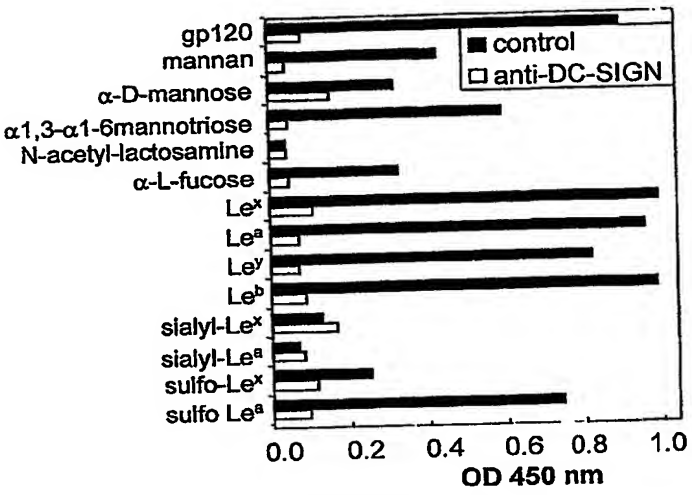
Abstract

C-type lectins are involved in the binding of many different types of carbohydrates. Considering their diversity in kind and expression of different types of cells, the influence of such binding is very diverse and dependent among others on the type of cell, the environment of the cell and the type of carbohydrate bound. In the present invention new carbohydrate specificities of C-type lectins are disclosed. Interference with this binding property has uses in the prevention of pathogen binding and also in influencing signaling pathways in the C-type lectin containing cell, particularly in Toll like receptor expressing cells such as dendritic cells. Also provided is the use of the carbohydrate specificity to enhance antigen presentation by antigen presenting cells and to manipulate migration of C-type lectin containing cells and the interaction of C-type lectin expressing cells with cellular ligands on neighboring cells.

Figure 1

A Is table of structures

B



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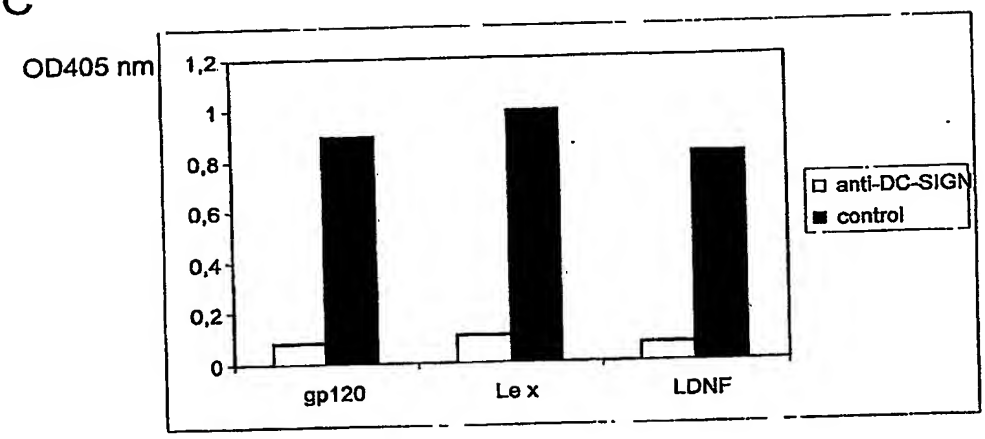


D

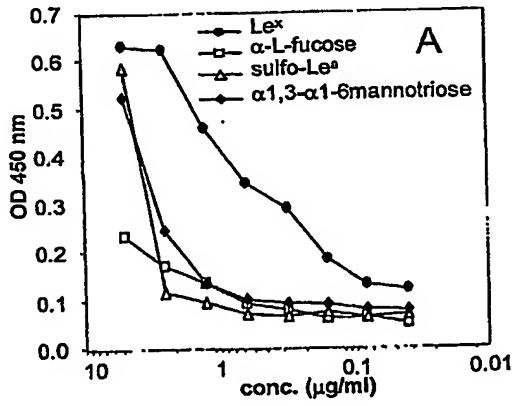


Figure 2

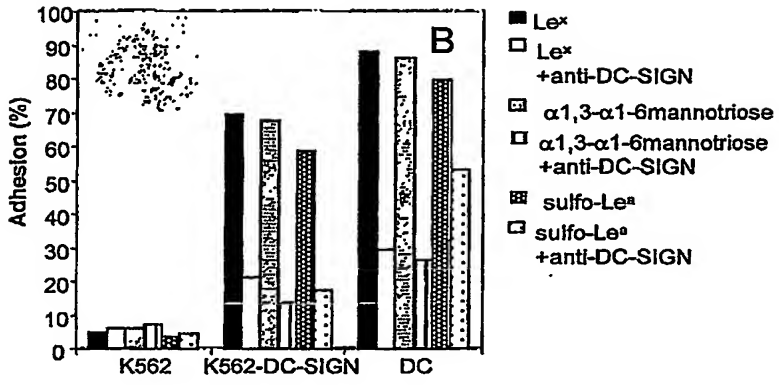
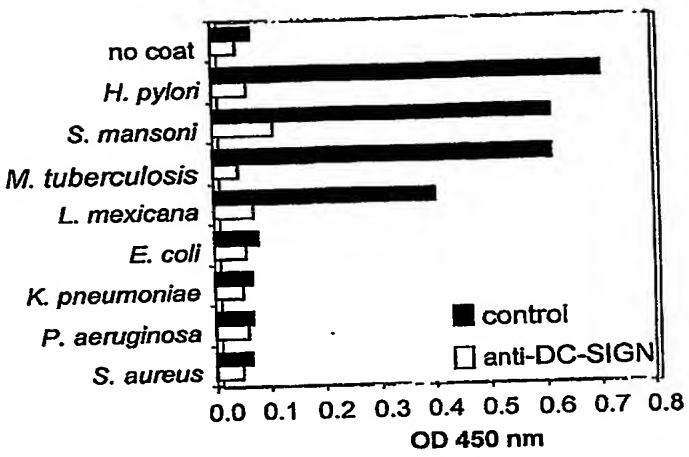

B
Adhesion (%)
100
90
80
70
60
50
40
30
20
10
0
K562
K562-DC-SIGN
DC
Le^x
Le^x +anti-DC-SIGN
α1,3-α1-6mannotriose
α1,3-α1-6mannotriose +anti-DC-SIGN
sulfo-Le^a
sulfo-Le^a +anti-DC-SIGN

Figure 3

A


no coat
H. pylori
S. mansoni
M. tuberculosis
L. mexicana
E. coli
K. pneumoniae
P. aeruginosa
S. aureus
control
anti-DC-SIGN
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
OD 450 nm

B

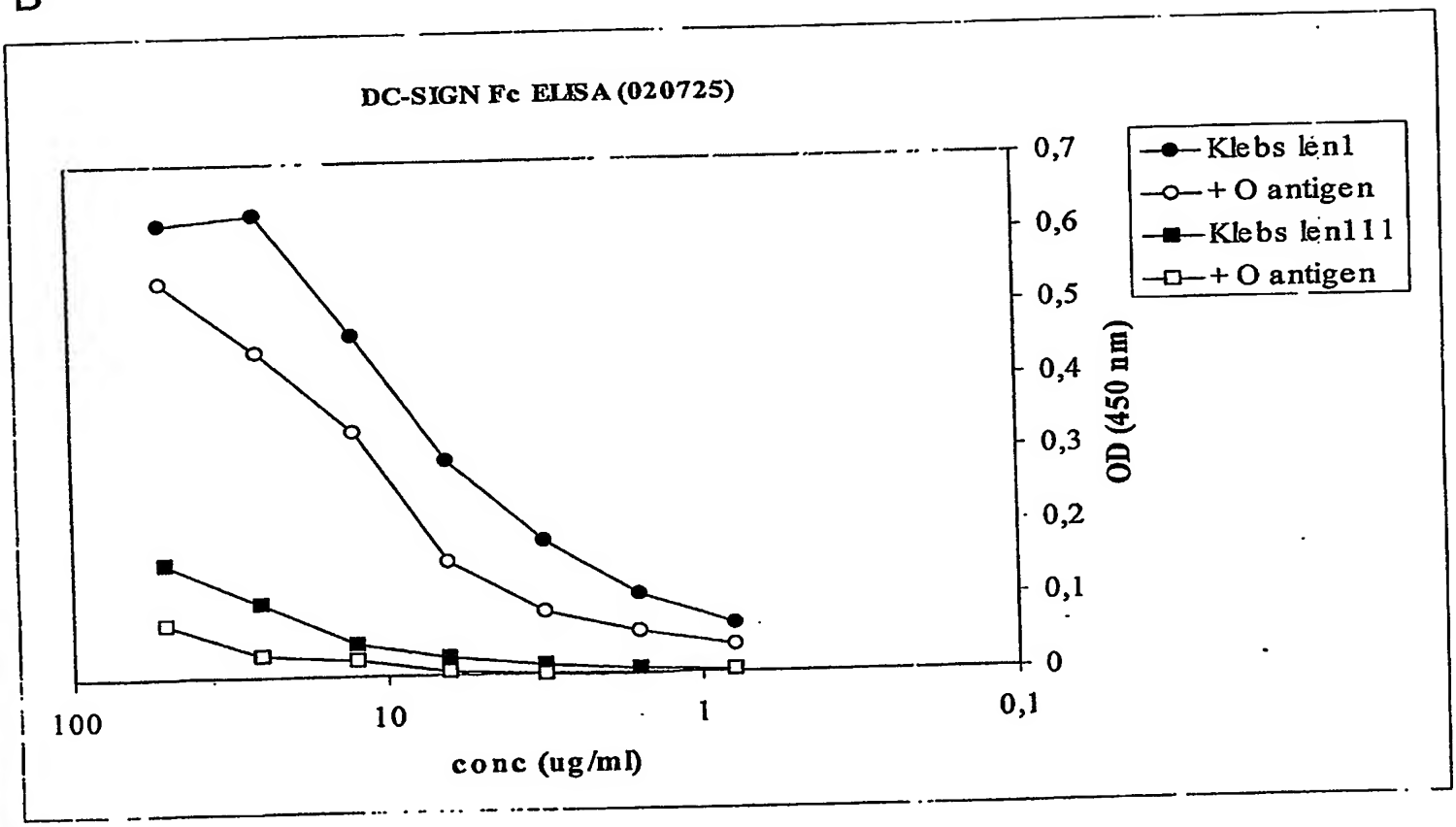

DC-SIGN Fc ELISA (020725)
Klebs len1
+ O antigen
Klebs len111
+ O antigen
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
OD (450 nm)
100
10
1
0,1
conc (ug/ml)

Figure 4

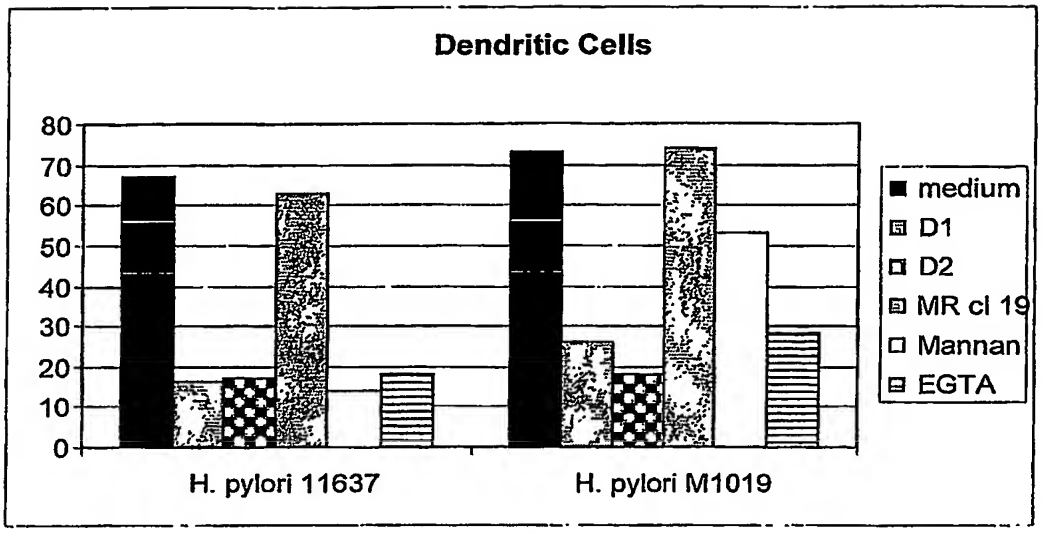

Dendritic Cells
80
70
60
50
40
30
20
10
0
H. pylori 11637
H. pylori M1019
medium
D1
D2
MR cl 19
Mannan
EGTA

Figure 5

Green: CD107a
Red: Le^X-PAA-bio

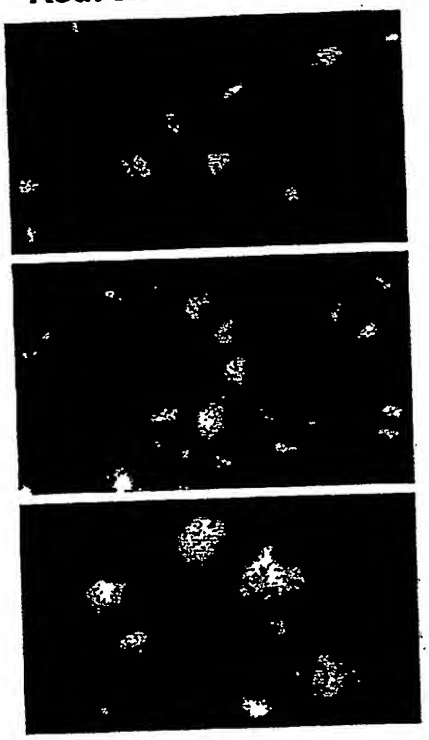


Figure 6

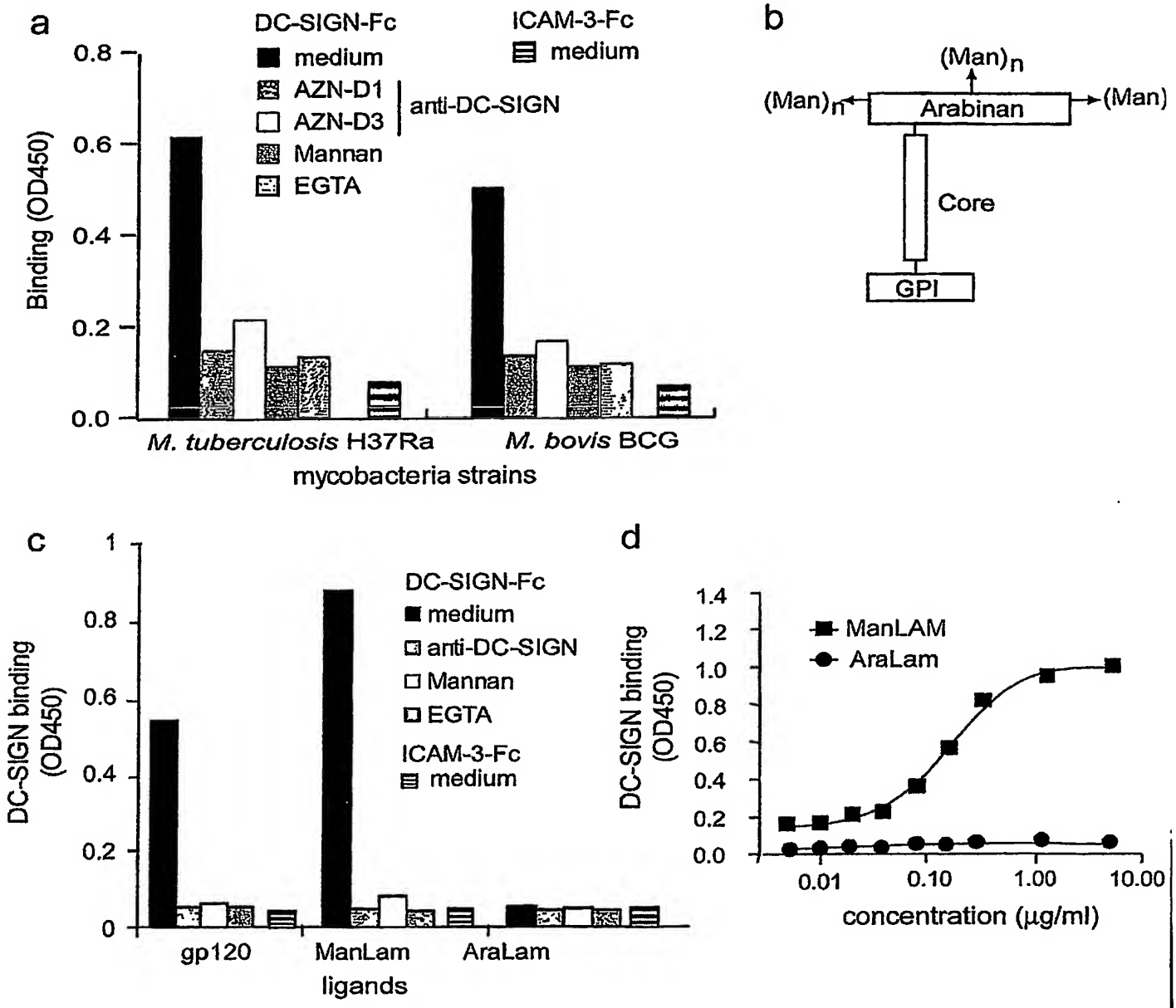

a
DC-SIGN-Fc
medium
AZN-D1
AZN-D3
anti-DC-SIGN
Mannan
EGTA
ICAM-3-Fc
medium
Binding (OD450)
0.8
0.6
0.4
0.2
0.0
M. tuberculosis H37Ra
M. bovis BCG
mycobacteria strains
b
(Man)n
(Man)n
Arabinan
(Man)
Core
GPI
c
DC-SIGN binding (OD450)
1
0.8
0.6
0.4
0.2
0
DC-SIGN-Fc
medium
anti-DC-SIGN
Mannan
EGTA
ICAM-3-Fc
medium
gp120
ManLam
AraLam
ligands
d
DC-SIGN binding (OD450)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ManLAM
AraLam
0.01
0.10
1.00
10.00
concentration (µg/ml)

Figure 7

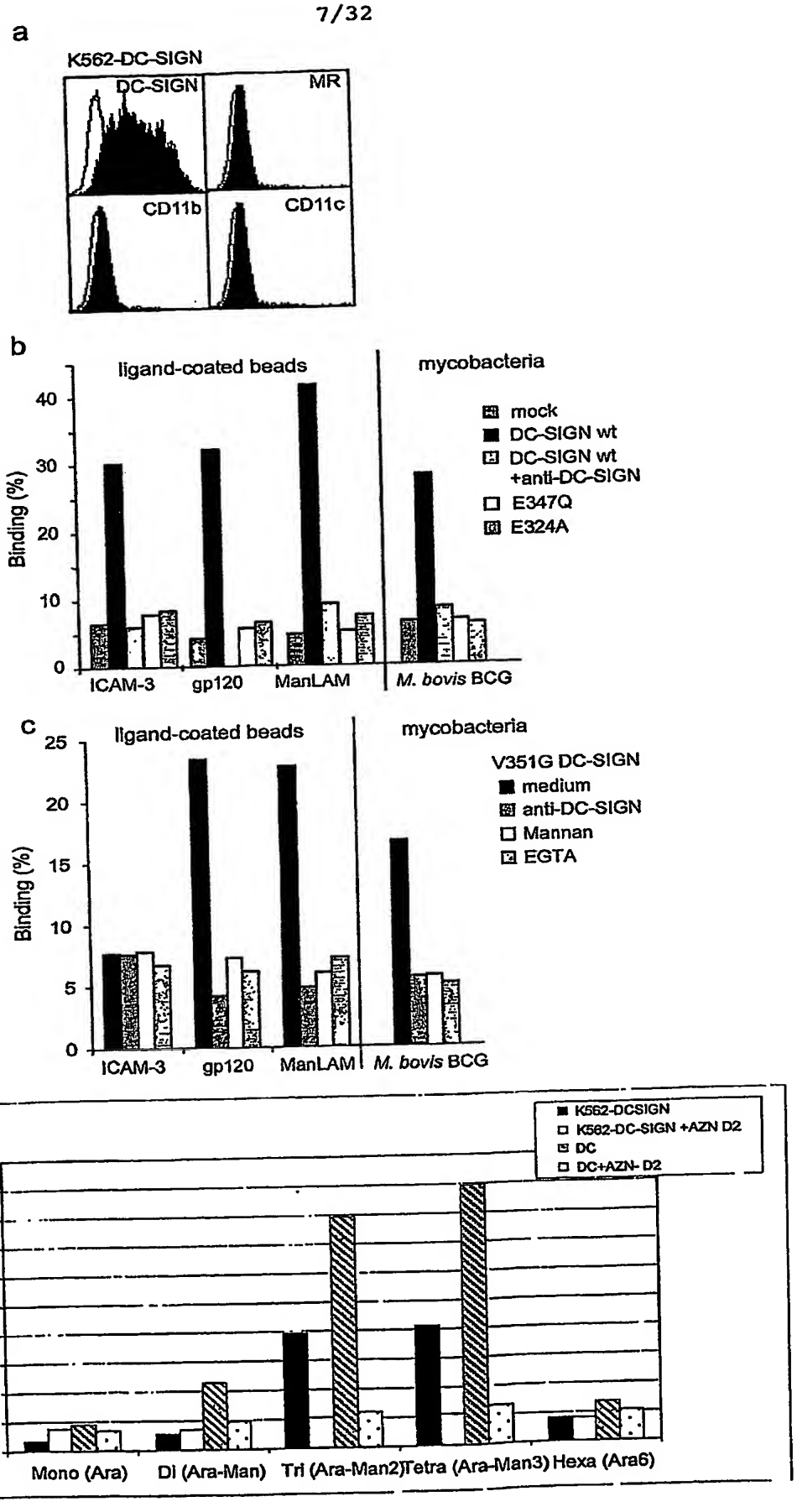

a
K562-DC-SIGN
DC-SIGN
MR
CD11b
CD11c
b
ligand-coated beads
mycobacteria
Binding (%)
mock
DC-SIGN wt
DC-SIGN wt +anti-DC-SIGN
E347Q
E324A
ICAM-3
gp120
ManLAM
M. bovis BCG
c
ligand-coated beads
mycobacteria
V351G DC-SIGN
medium
anti-DC-SIGN
Mannan
EGTA
Binding (%)
ICAM-3
gp120
ManLAM
M. bovis BCG
d
K562-DCSIGN
K562-DC-SIGN +AZN D2
DC
DC+AZN- D2
Binding (%)
Mono (Ara)
DI (Ara-Man)
Tri (Ara-Man2)
Tetra (Ara-Man3)
Hexa (Ara6)

Figure 8

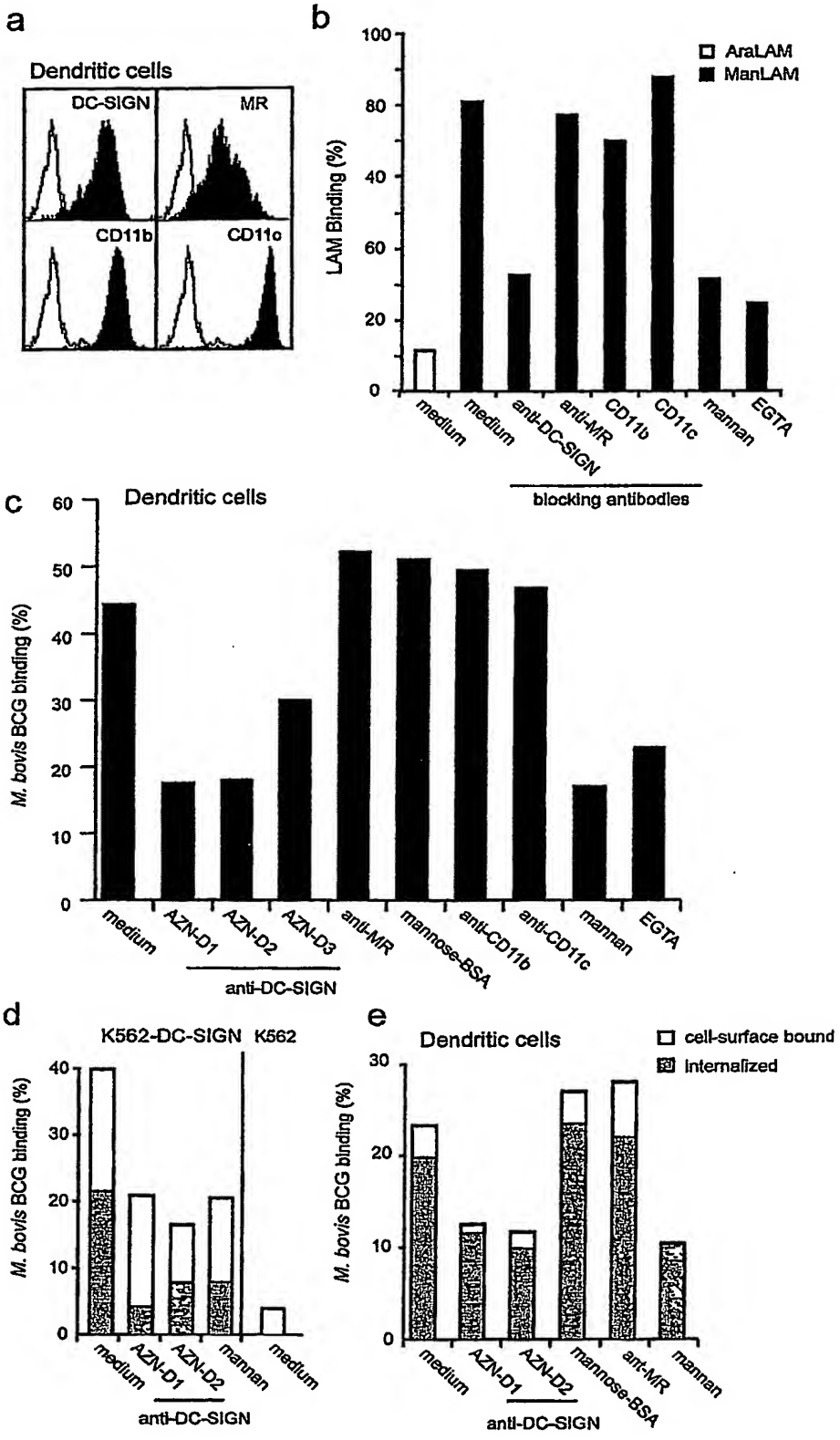
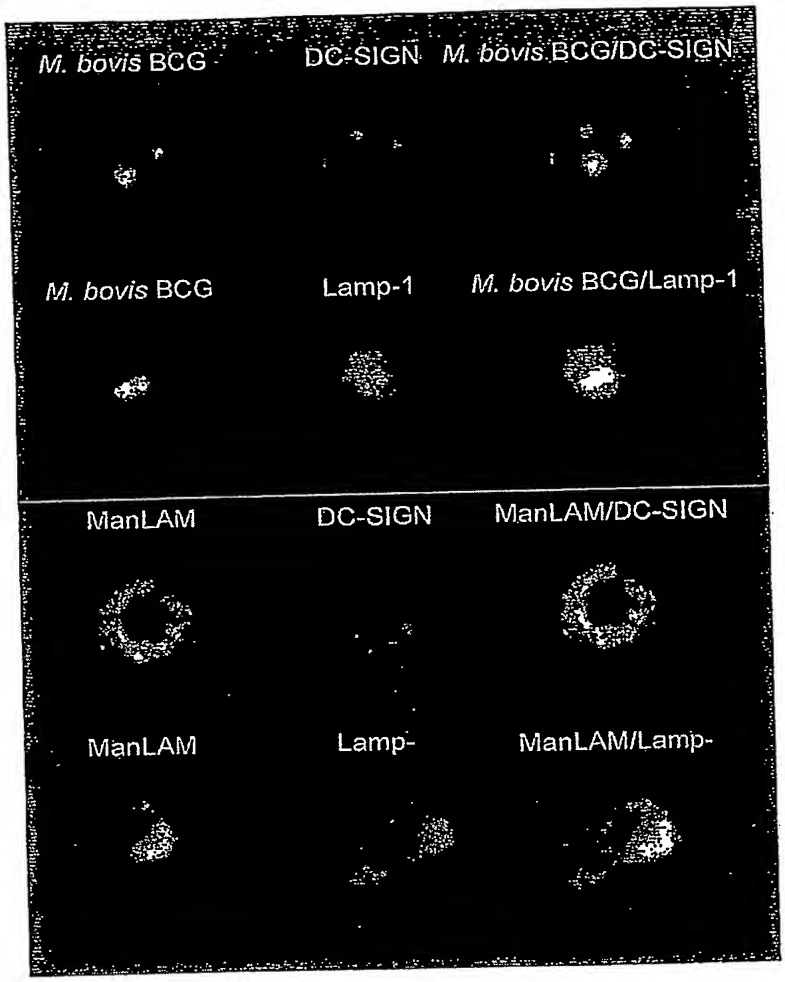

a
Dendritic cells
DC-SIGN
MR
CD11b
CD11c
b
AraLAM
ManLAM
LAM Binding (%)
100
80
60
40
20
0
medium
medium
anti-DC-SIGN
anti-MR
CD11b
CD11c
mannan
EGTA
blocking antibodies
c
Dendritic cells
M. bovis BCG binding (%)
60
50
40
30
20
10
0
medium
AZN-D1
AZN-D2
AZN-D3
anti-MR
mannose-BSA
anti-CD11b
anti-CD11c
mannan
EGTA
anti-DC-SIGN
d
K562-DC-SIGN
K562
M. bovis BCG binding (%)
40
30
20
10
0
medium
AZN-D1
AZN-D2
mannan
medium
anti-DC-SIGN
e
Dendritic cells
cell-surface bound
internalized
M. bovis BCG binding (%)
30
20
10
0
medium
AZN-D1
AZN-D2
mannose-BSA
anti-MR
mannan
anti-DC-SIGN

Figure 9

a


M. bovis BCG
DC-SIGN
M. bovis BCG/DC-SIGN
M. bovis BCG
Lamp-1
M. bovis BCG/Lamp-1
ManLAM
DC-SIGN
ManLAM/DC-SIGN
ManLAM
Lamp-
ManLAM/Lamp-

b

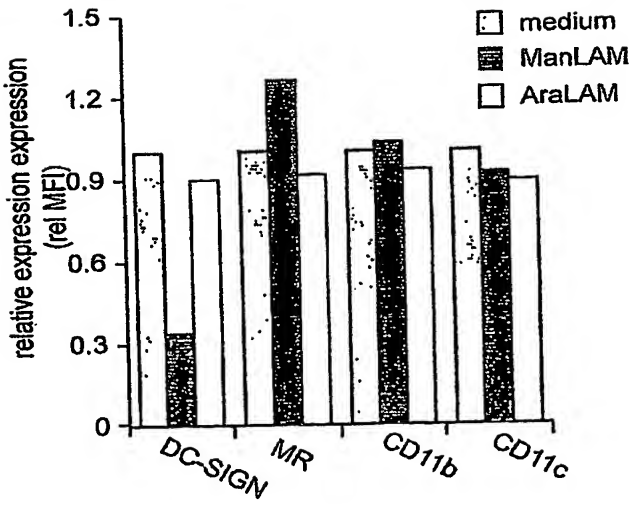

1.5
1.2
0.9
0.6
0.3
0
relative expression expression (rel MFI)
medium
ManLAM
AraLAM
DC-SIGN
MR
CD11b
CD11c

Figure 10

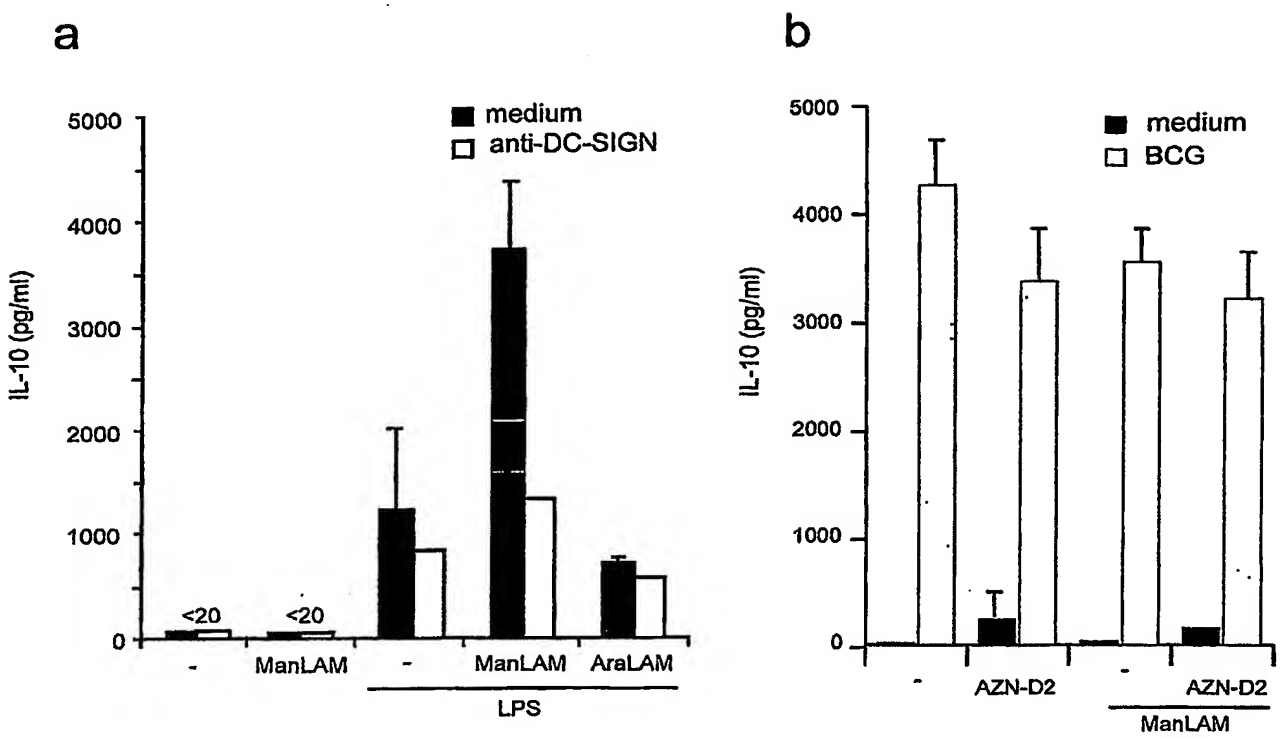

a
5000
4000
3000
2000
1000
0
IL-10 (pg/ml)
medium
anti-DC-SIGN
<20
<20
-
ManLAM
-
ManLAM
AraLAM
LPS
b
5000
4000
3000
2000
1000
0
IL-10 (pg/ml)
medium
BCG
-
AZN-D2
-
AZN-D2
ManLAM

Figure 11

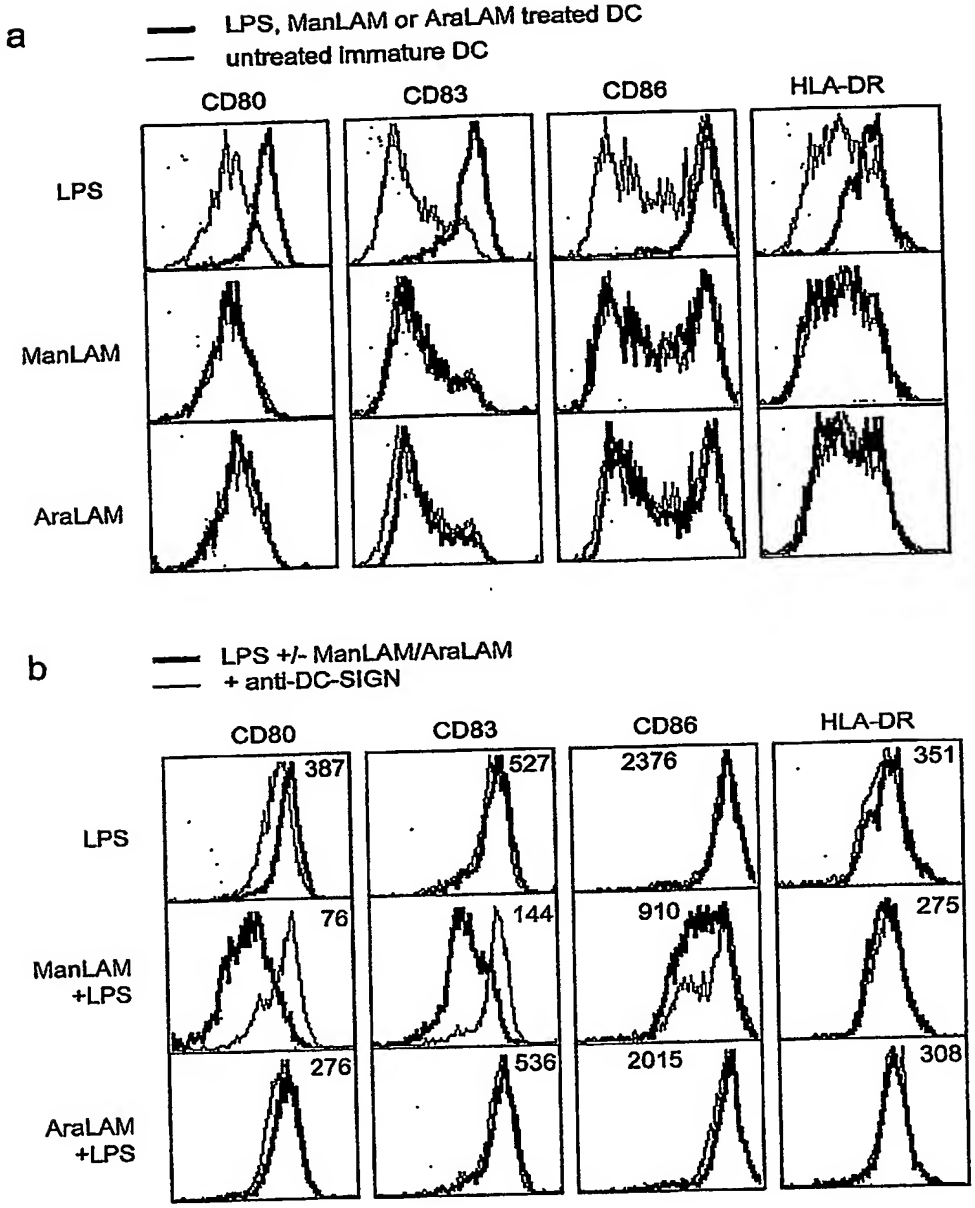
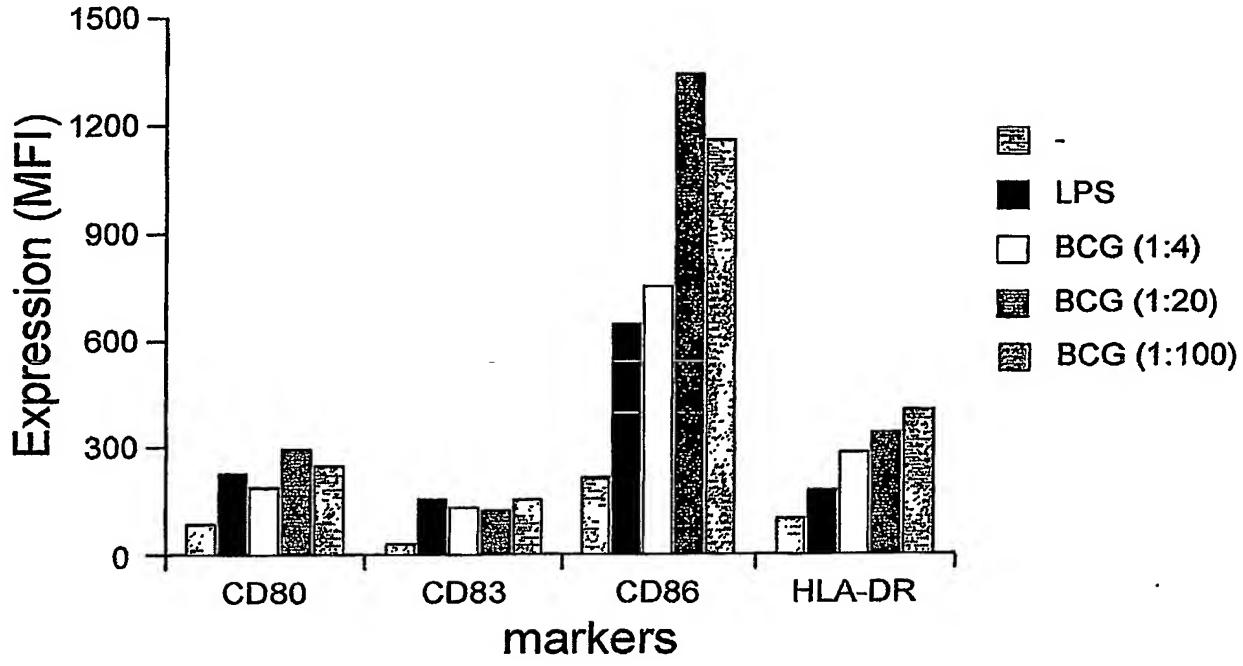

a
LPS, ManLAM or AraLAM treated DC
untreated Immature DC
CD80
CD83
CD86
HLA-DR
LPS
ManLAM
AraLAM
b
LPS +/- ManLAM/AraLAM
+ anti-DC-SIGN
CD80
CD83
CD86
HLA-DR
387
527
2376
351
LPS
76
144
910
275
ManLAM +LPS
276
536
2015
308
AraLAM +LPS

Figure 12

a


1500
1200
900
600
300
0
Expression (MFI)
CD80
CD83
CD86
HLA-DR
markers
-
LPS
BCG (1:4)
BCG (1:20)
BCG (1:100)

b

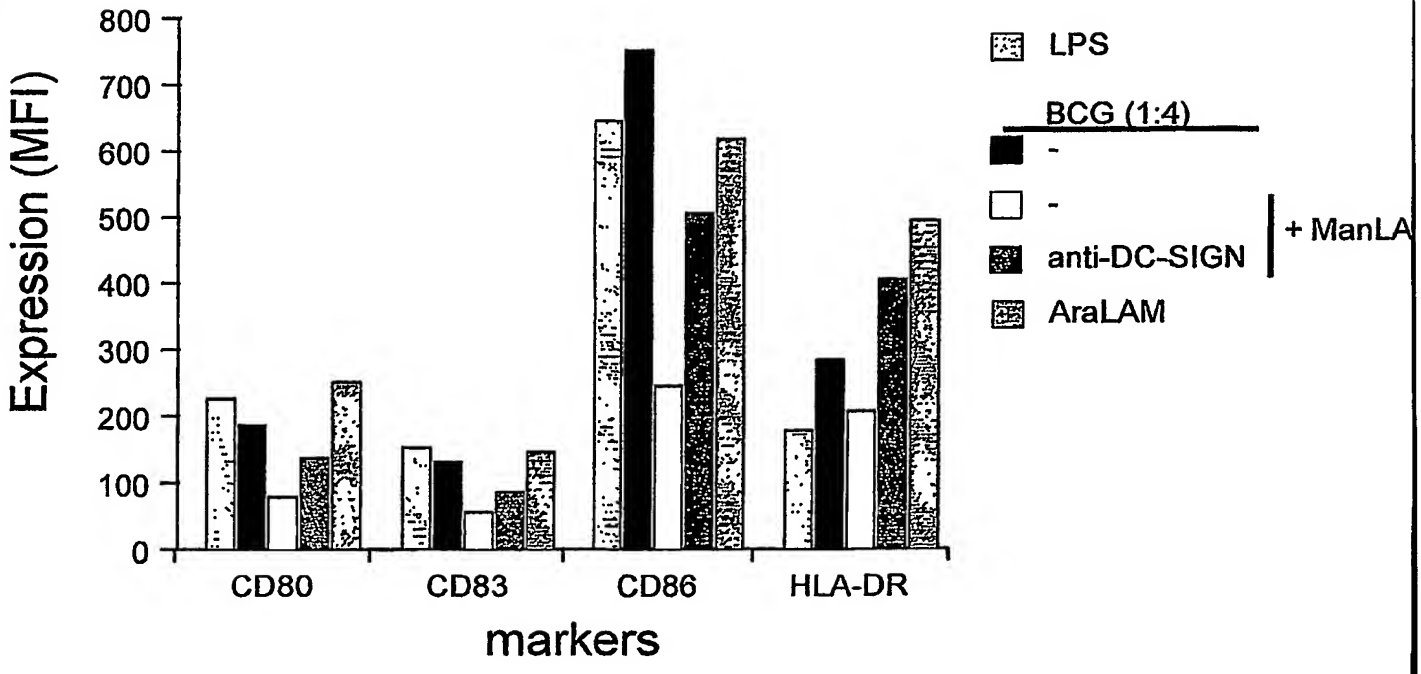

800
700
600
500
400
300
200
100
0
Expression (MFI)
CD80
CD83
CD86
HLA-DR
markers
LPS
BCG (1:4)
-
-
anti-DC-SIGN
AraLAM
+ ManLA

Figure 13

Carbohydrate antigens on *S. mansoni* SEA

Carbohydrate epitope	Shortname	anti-glycan MAb
Gal β4 (α3) GlcNAc R	Lewis-X	CB10 (32)
GalNAc β4 GlcNAc R	LDN	SMLDN1.1 (31)
GalNAc β4 (α3) GlcNAc R	LDNF	SMLDNF1 (4)
GalNAc β4 (α2, α3) GlcNAc R	LDN-DF	114-5B1-A (12)

Gal ■ GlcNAc ▲ GalNAc ◆ Fucose

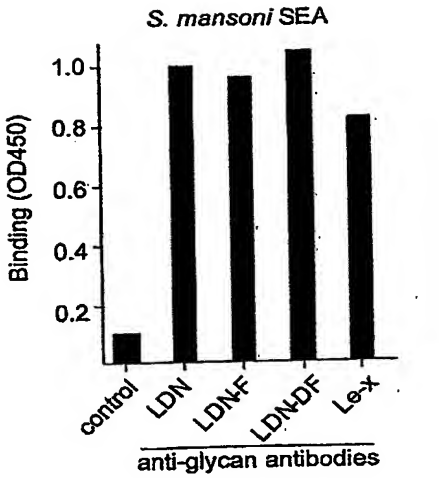


Figure 14

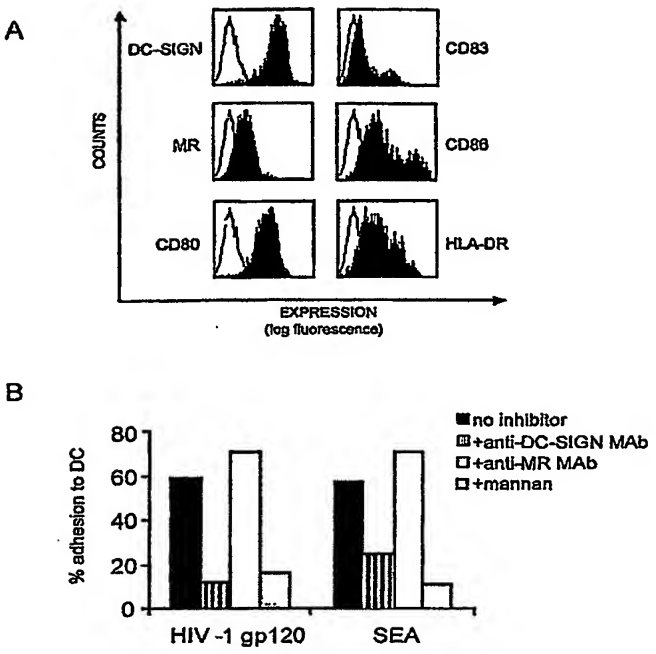

A
DC-SIGN
CD83
MR
CD86
CD80
HLA-DR
COUNTS
EXPRESSION
(log fluorescence)
B
no inhibitor
+anti-DC-SIGN MAb
+anti-MR MAb
+mannan
% adhesion to DC
80
60
40
20
0
HIV -1 gp120
SEA

Figure 15

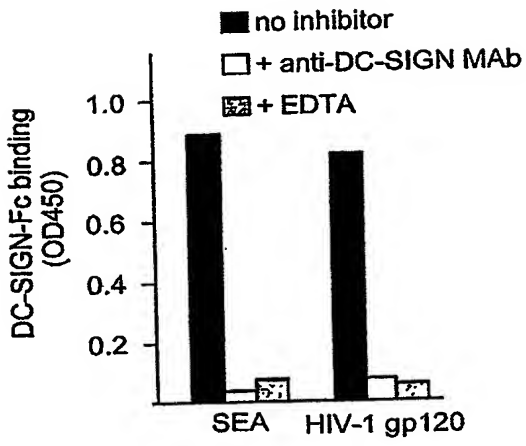

no inhibitor
+ anti-DC-SIGN MAb
+ EDTA
DC-SIGN-Fc binding (OD450)
1.0
0.8
0.6
0.4
0.2
SEA
HIV-1 gp120

Figure 16

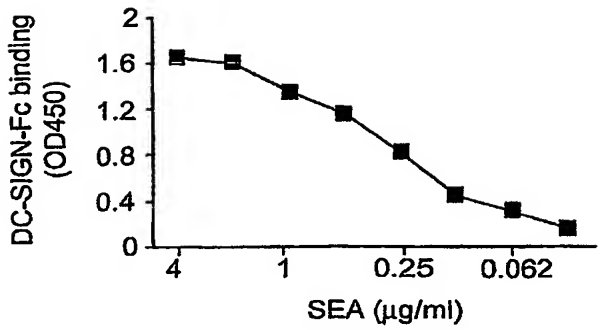
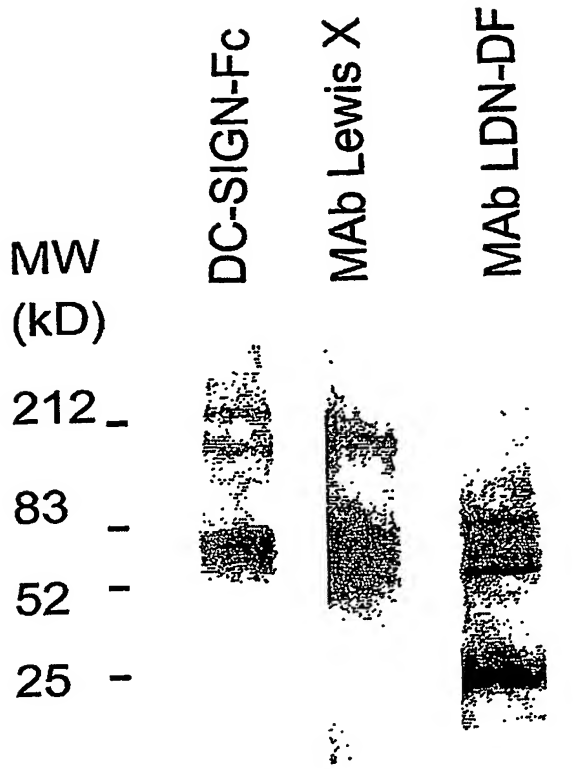


Figure 17



S. mansoni SEA

Fig. 18

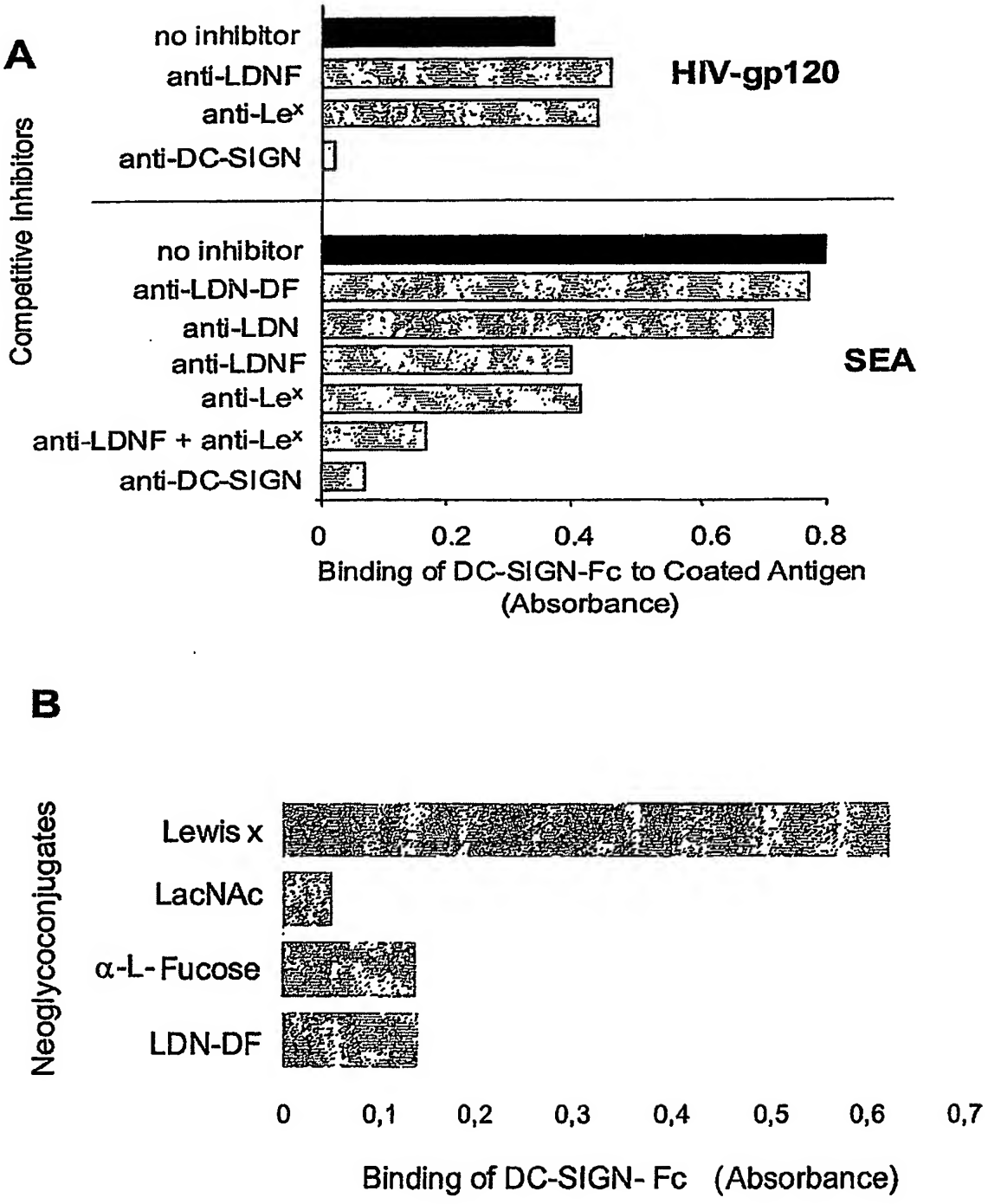

A
Competitive Inhibitors
no inhibitor
anti-LDNF
anti-Le^x
anti-DC-SIGN
HIV-gp120
no inhibitor
anti-LDN-DF
anti-LDN
anti-LDNF
anti-Le^x
anti-LDNF + anti-Le^x
anti-DC-SIGN
SEA
0
0.2
0.4
0.6
0.8
Binding of DC-SIGN-Fc to Coated Antigen
(Absorbance)
B
Neoglycoconjugates
Lewis x
LacNAc
α-L-Fucose
LDN-DF
0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
Binding of DC-SIGN- Fc (Absorbance)

Figure 19

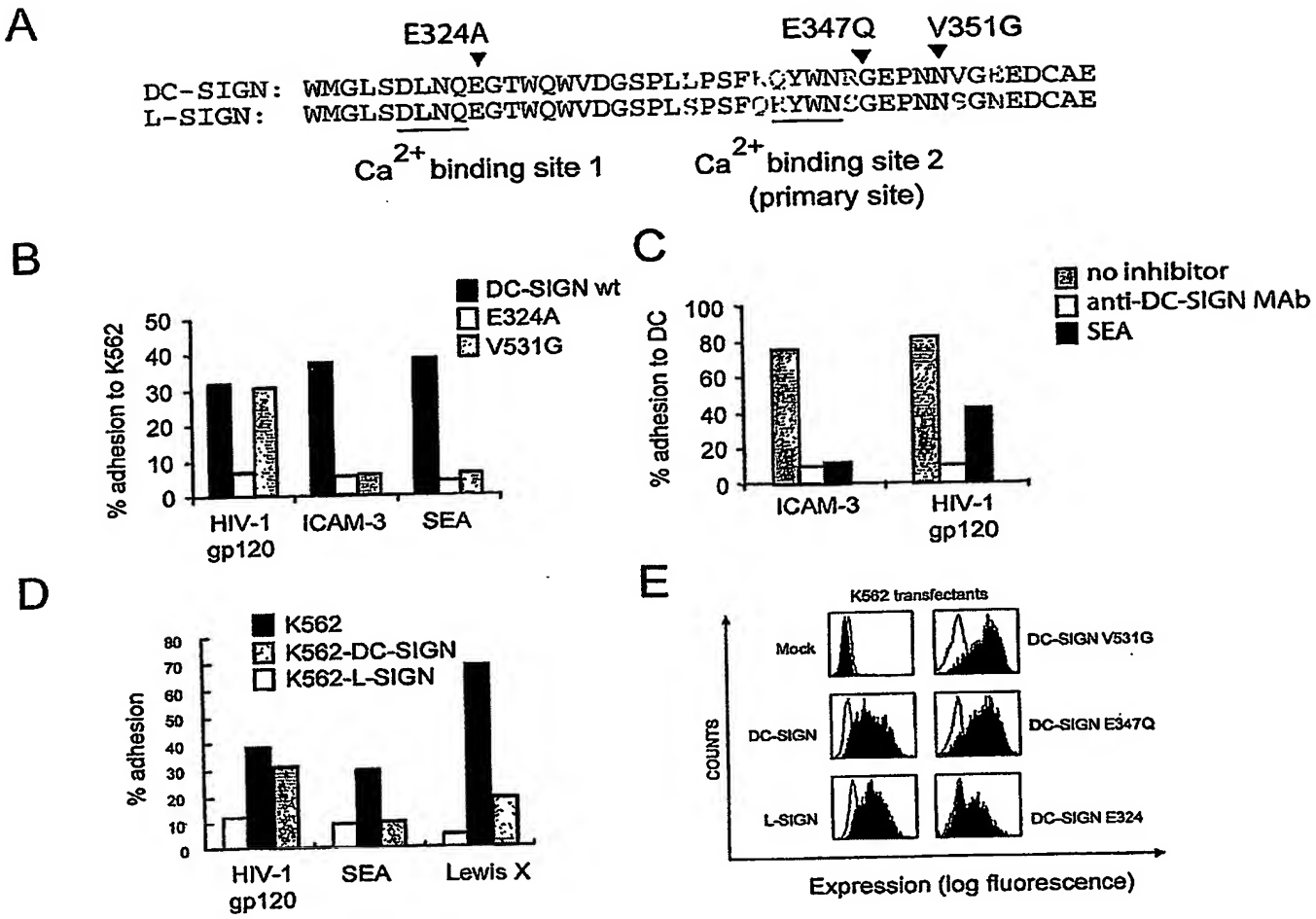
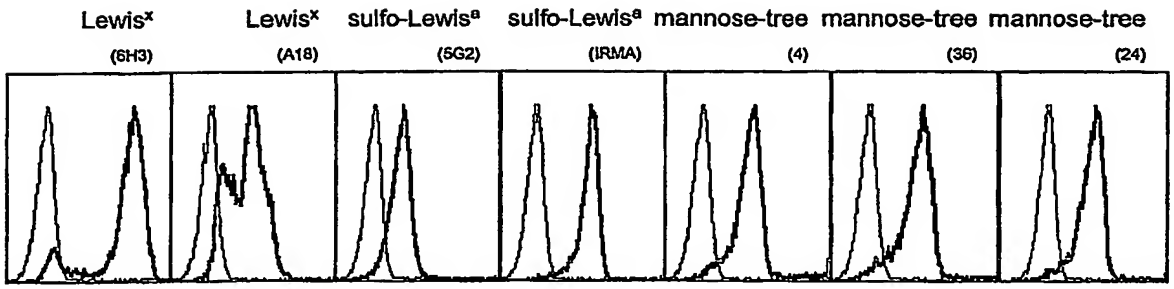

A
E324A
E347Q
V351G
DC-SIGN: WMGLSDLNQEGTWQWVDGSPLLPSFKQYWNRGEPNNVGEEDCAE
L-SIGN: WMGLSDLNQEGTWQWVDGSPLSPSFQRYWNSGEPNNSGNEDCAE
Ca2+ binding site 1
Ca2+ binding site 2 (primary site)
B
DC-SIGN wt
E324A
V531G
% adhesion to K562
HIV-1 gp120
ICAM-3
SEA
C
no inhibitor
anti-DC-SIGN MAb
SEA
% adhesion to DC
ICAM-3
HIV-1 gp120
D
K562
K562-DC-SIGN
K562-L-SIGN
% adhesion
HIV-1 gp120
SEA
Lewis X
E
K562 transfectants
Mock
DC-SIGN
L-SIGN
DC-SIGN V531G
DC-SIGN E347Q
DC-SIGN E324
COUNTS
Expression (log fluorescence)

Figure 20

A


Lewis^x (6H3)
Lewis^x (A18)
sulfo-Lewis^a (5G2)
sulfo-Lewis^a (IRMA)
mannose-tree (4)
mannose-tree (36)
mannose-tree (24)

B

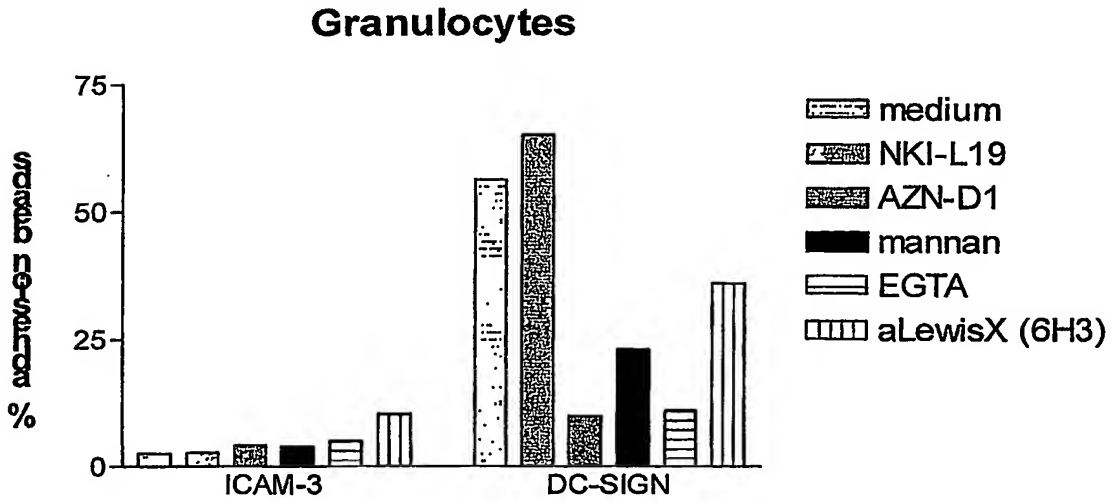
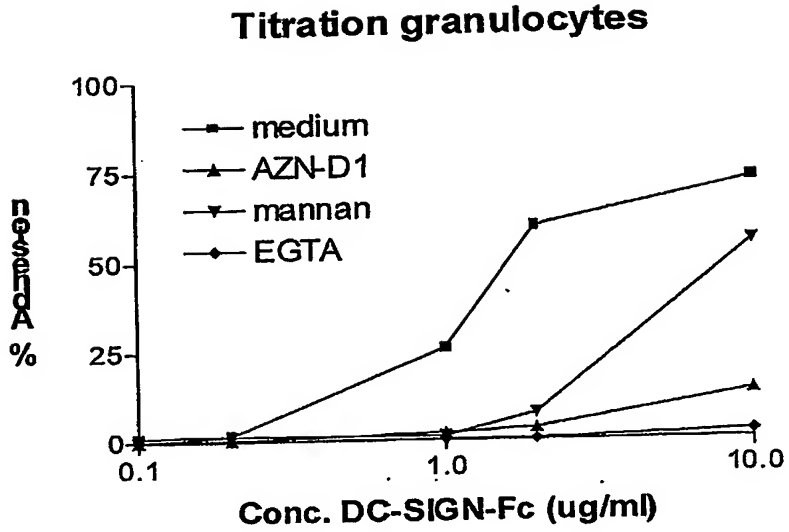

Granulocytes
75
50
25
0
%
ICAM-3
DC-SIGN
medium
NKI-L19
AZN-D1
mannan
EGTA
aLewisX (6H3)

Figure 21


Titration granulocytes
100
75
50
25
0
0.1
1.0
10.0
medium
AZN-D1
mannan
EGTA
%
Conc. DC-SIGN-Fc (ug/ml)

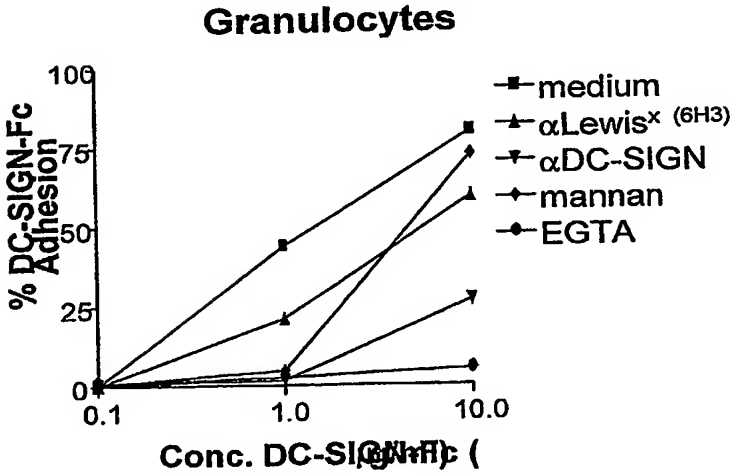
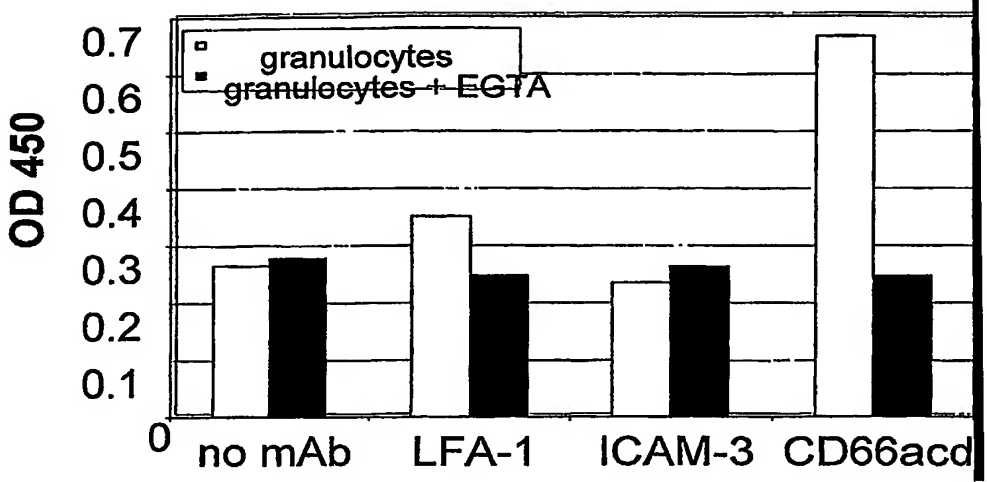
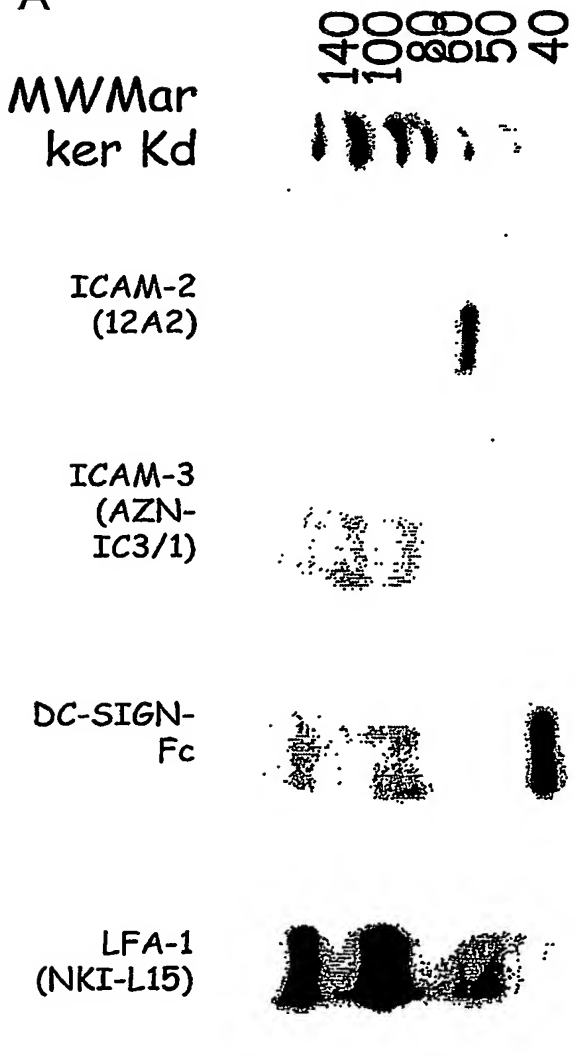

Granulocytes
100
75
50
25
0
0.1
1.0
10.0
% DC-SIGN-Fc Adhesion
medium
αLewis^{x} (6H3)
αDC-SIGN
mannan
EGTA

Figure 22

C



A



B

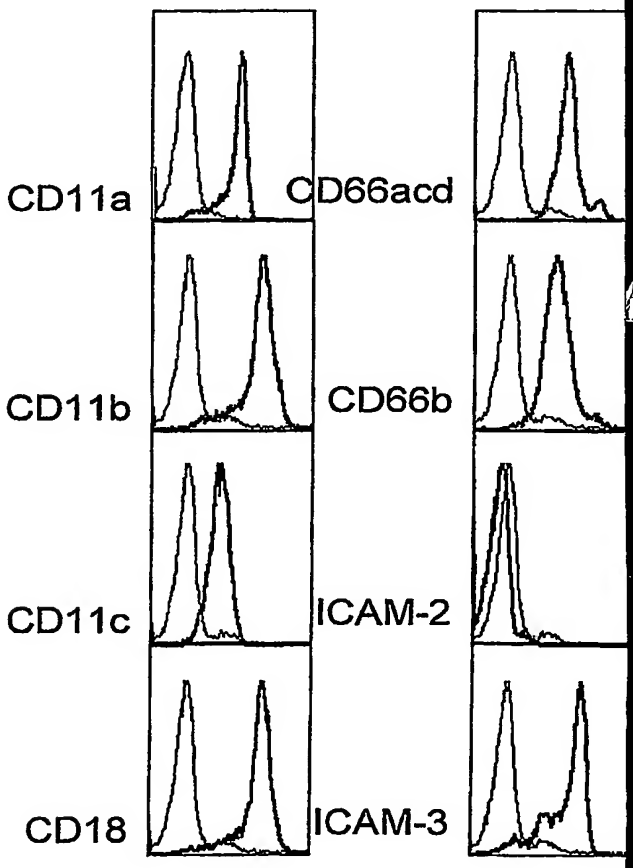


Figure 23

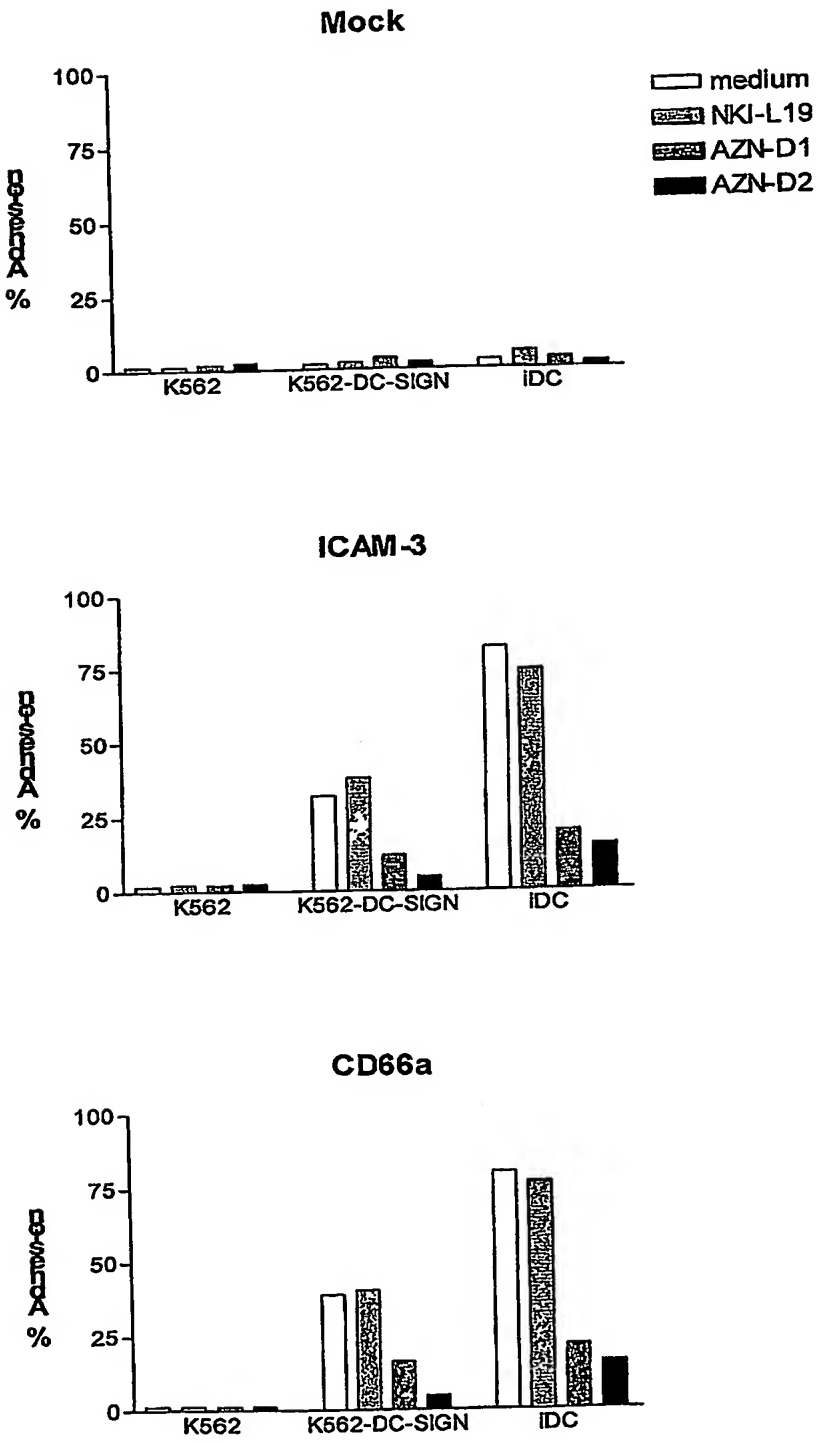

Mock
medium
NKI-L19
AZN-D1
AZN-D2
% cytotoxicity
100
75
50
25
0
K562
K562-DC-SIGN
iDC
ICAM-3
% cytotoxicity
100
75
50
25
0
K562
K562-DC-SIGN
iDC
CD66a
% cytotoxicity
100
75
50
25
0
K562
K562-DC-SIGN
iDC

Figure 24

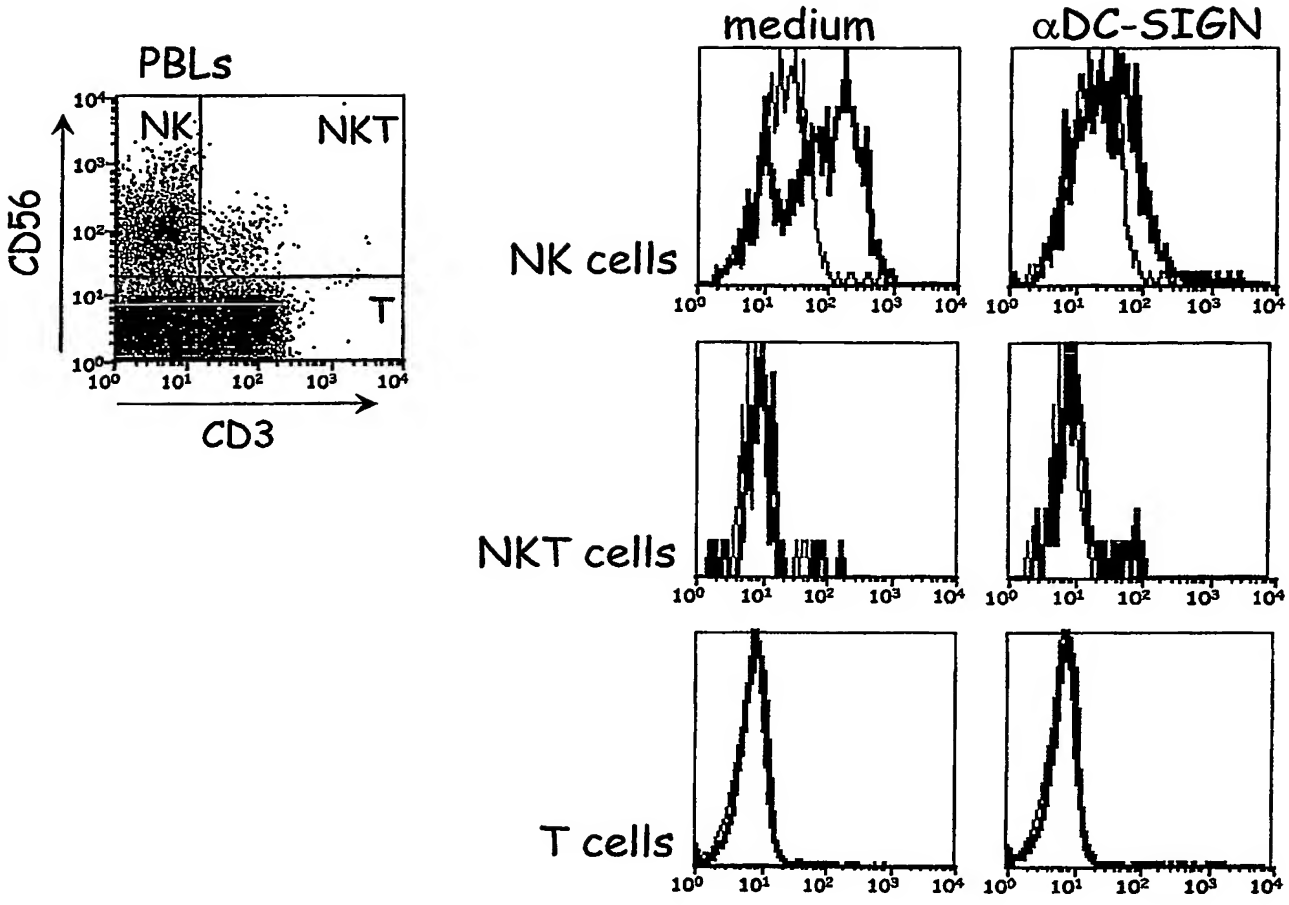

PBLs
NK
NKT
T
CD56
CD3
medium
αDC-SIGN
NK cells
NKT cells
T cells

Figure 25

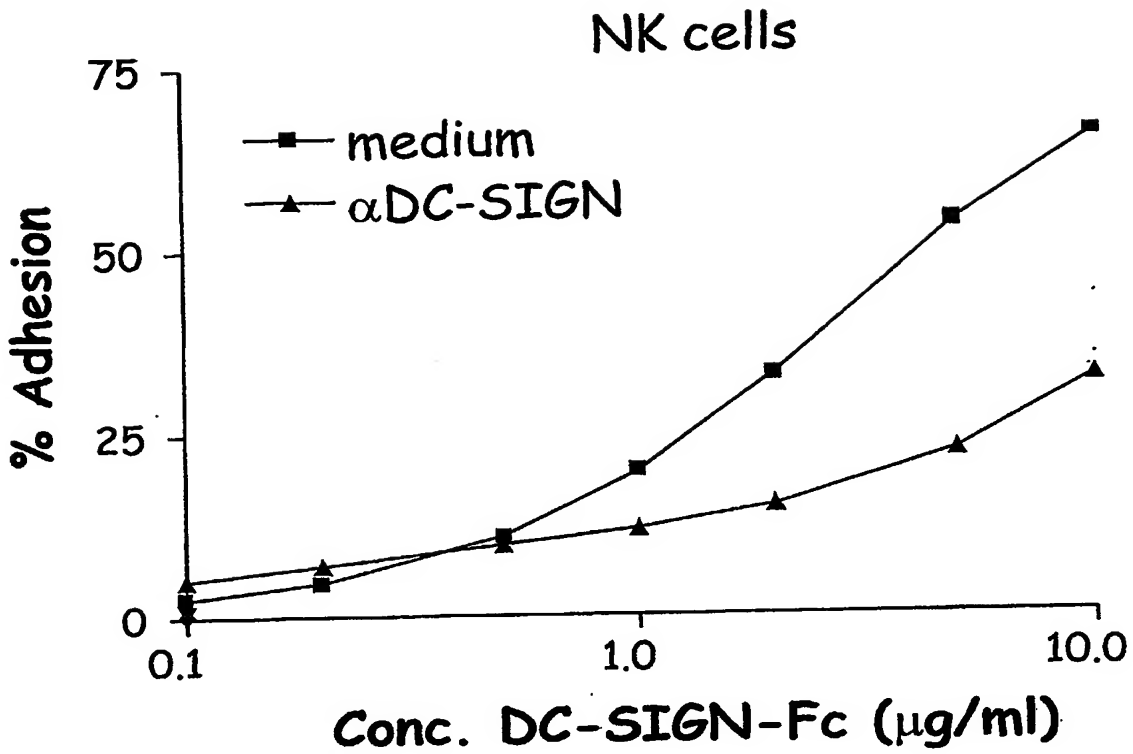

NK cells
75
50
25
0
% Adhesion
medium
αDC-SIGN
0.1
1.0
10.0
Conc. DC-SIGN-Fc (μg/ml)

Figure 26

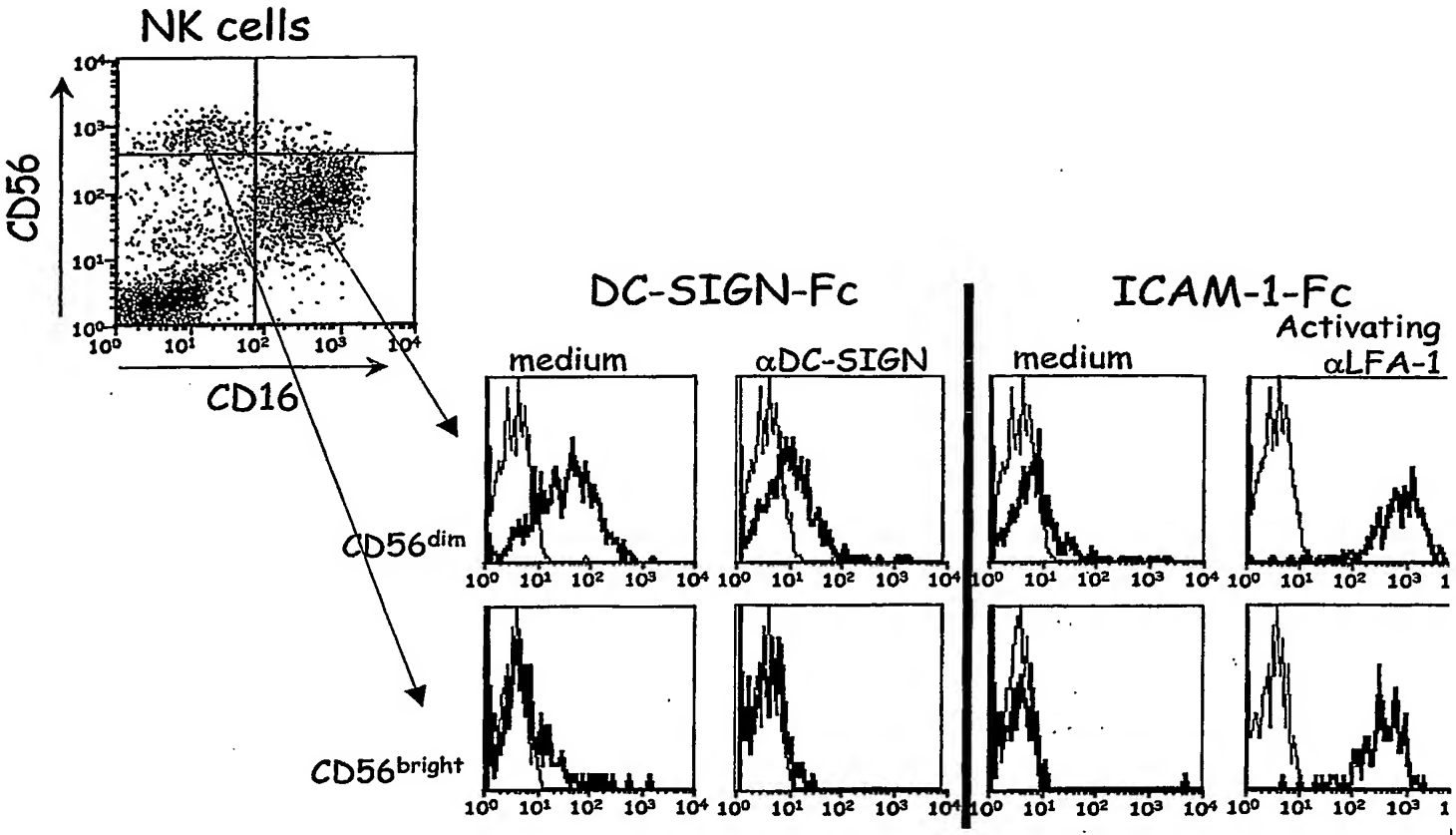
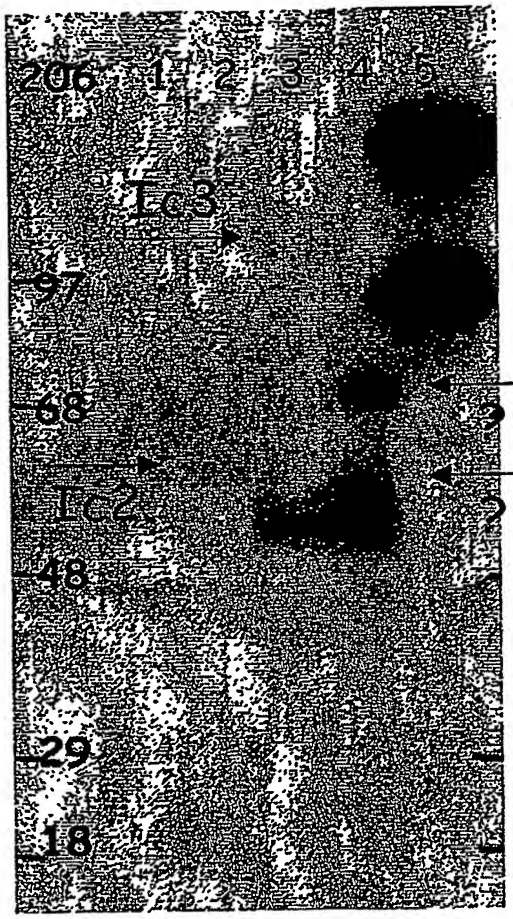

NK cells
CD56
CD16
DC-SIGN-Fc
ICAM-1-Fc
medium
αDC-SIGN
medium
Activating
αLFA-1
CD56dim
CD56bright

Figure 27



1. control
2. ICAM-2
3. ICAM-3
4. DC-SIGN ligand
5. LFA-1

→ DC-SIGN binds ICAM-2? and 75 kD ligand on NK cells

Figure 28

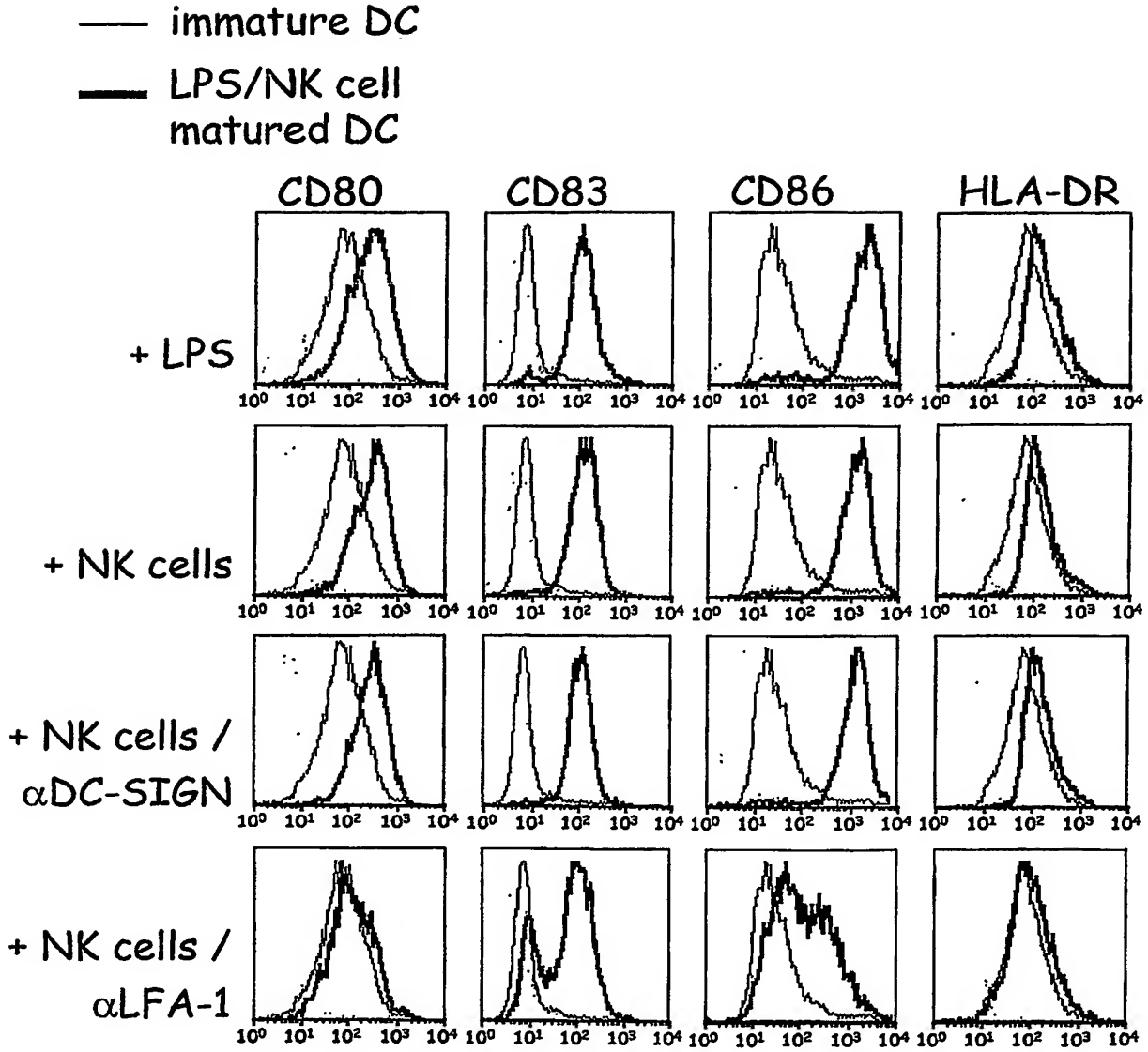

immature DC
LPS/NK cell matured DC
CD80
CD83
CD86
HLA-DR
+ LPS
+ NK cells
+ NK cells / αDC-SIGN
+ NK cells / αLFA-1
10⁰ 10¹ 10² 10³ 10⁴

Figure 29

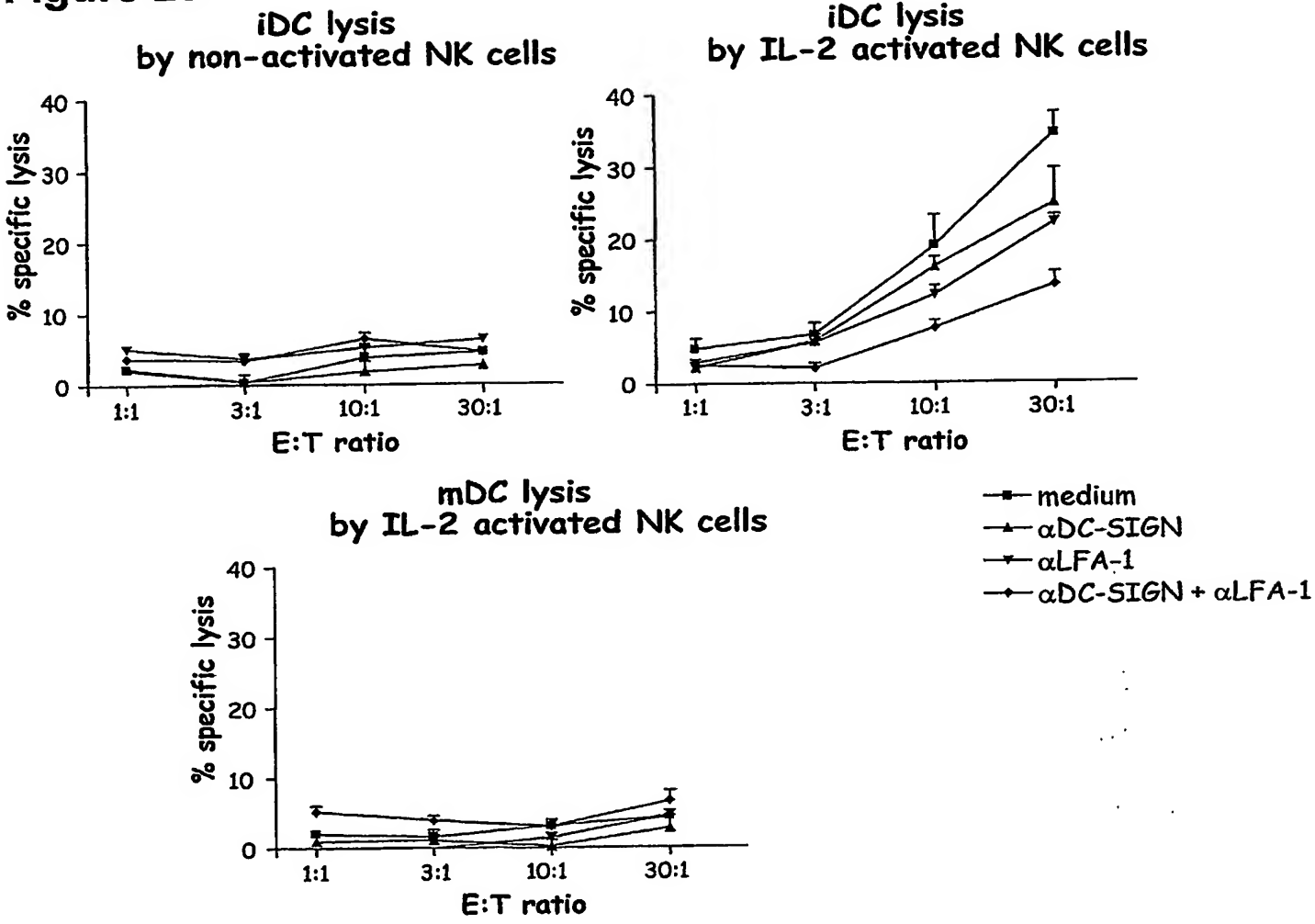

iDC lysis
by non-activated NK cells
% specific lysis
40
30
20
10
0
1:1
3:1
10:1
30:1
E:T ratio
iDC lysis
by IL-2 activated NK cells
% specific lysis
40
30
20
10
0
1:1
3:1
10:1
30:1
E:T ratio
mDC lysis
by IL-2 activated NK cells
% specific lysis
40
30
20
10
0
1:1
3:1
10:1
30:1
E:T ratio
medium
αDC-SIGN
αLFA-1
αDC-SIGN + αLFA-1

Figure 30

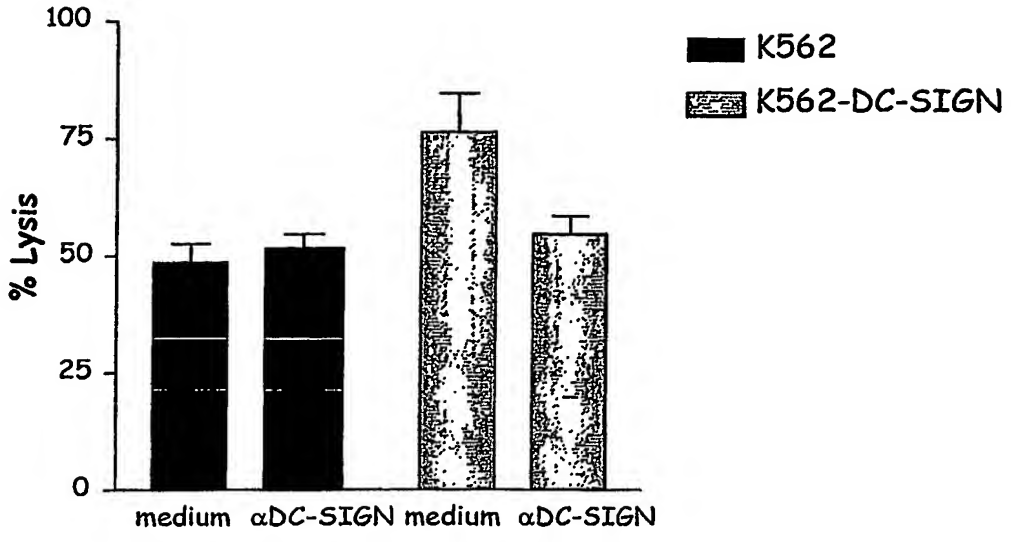

100
75
50
25
0
% Lysis
K562
K562-DC-SIGN
medium
αDC-SIGN
medium
αDC-SIGN

Figure 31

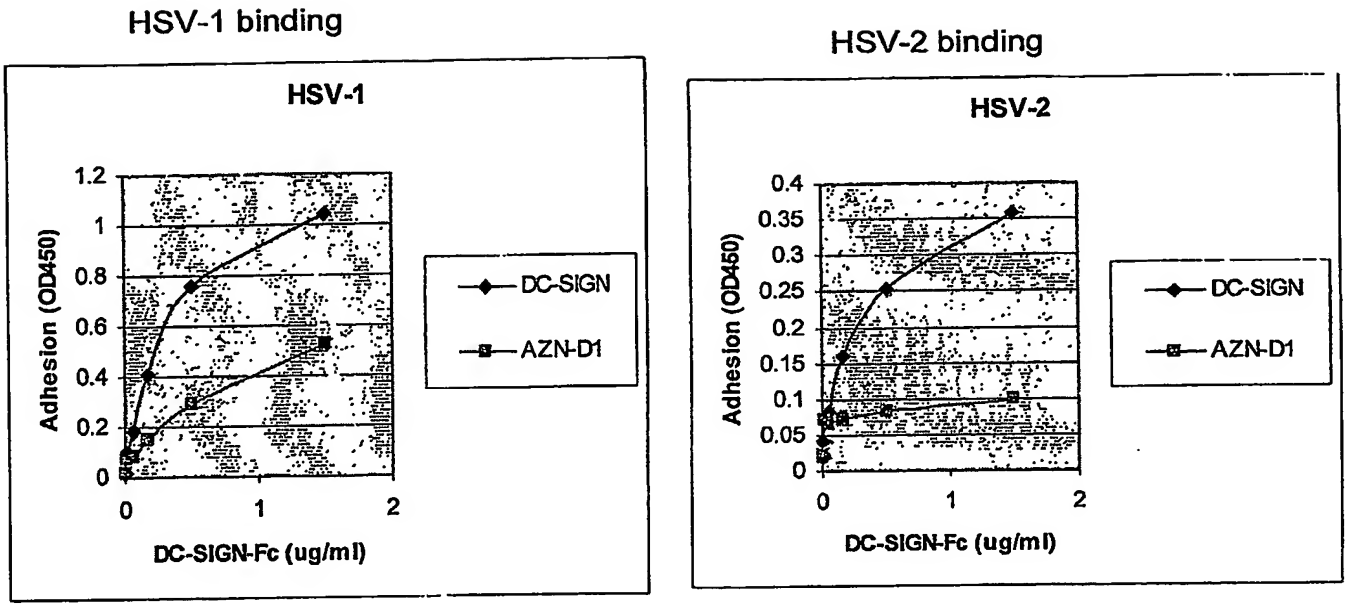
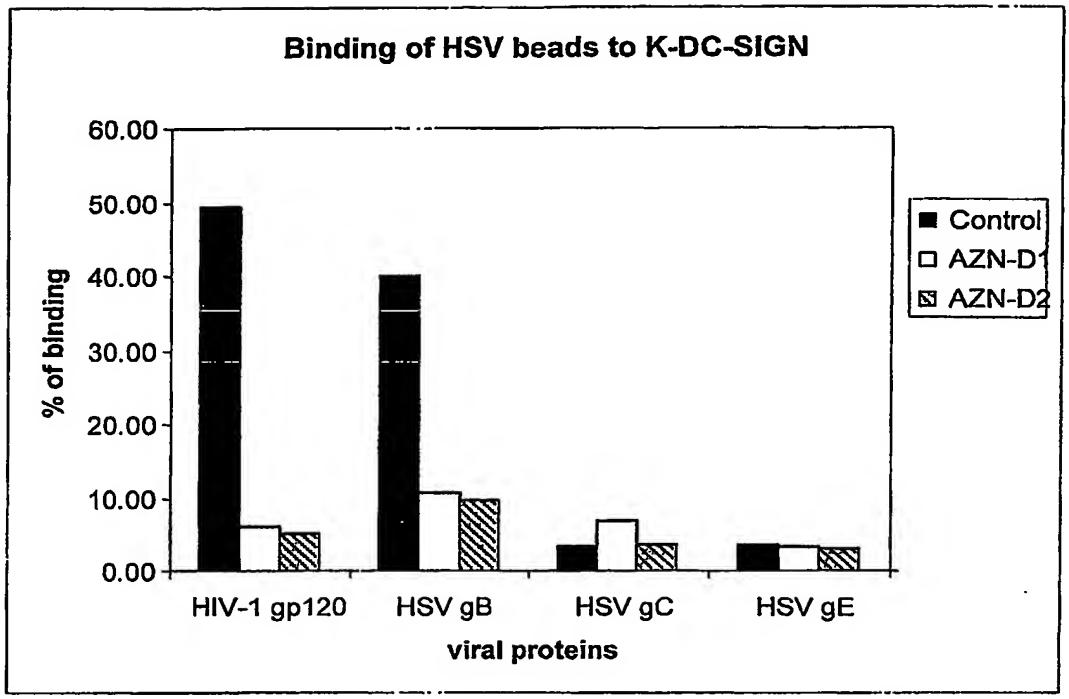
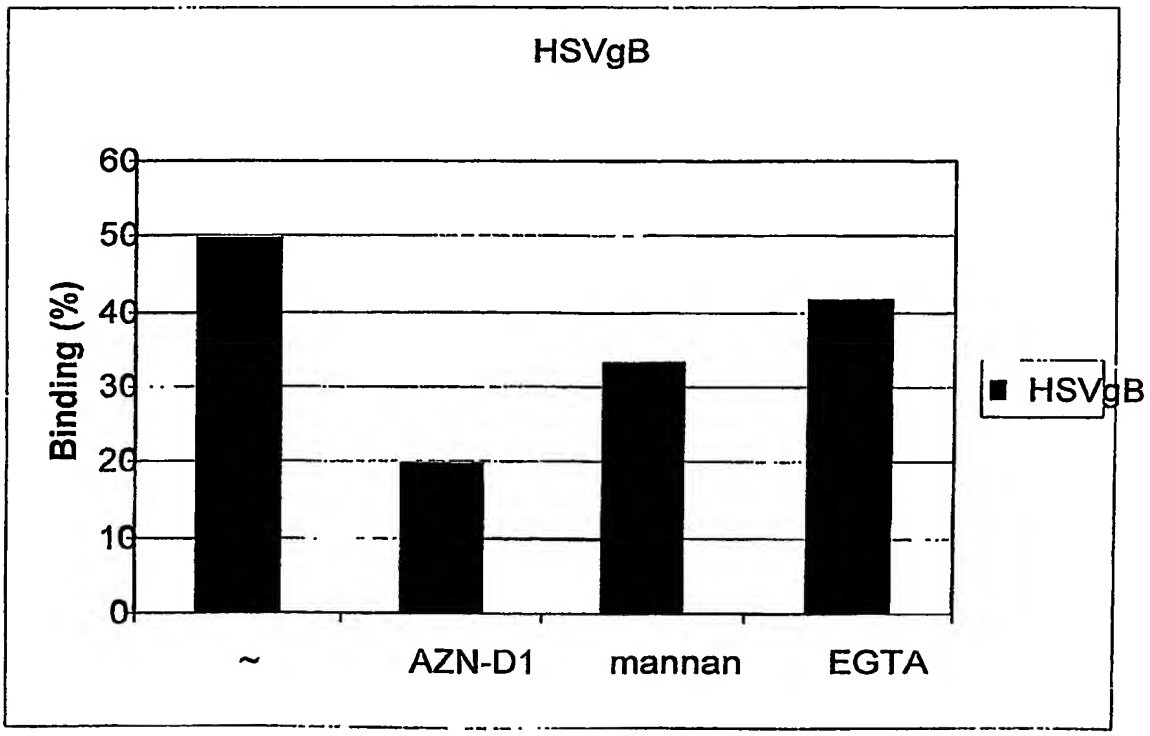

HSV-1 binding
HSV-1
Adhesion (OD450)
1.2
1
0.8
0.6
0.4
0.2
0
0
1
2
DC-SIGN-Fc (ug/ml)
DC-SIGN
AZN-D1
HSV-2 binding
HSV-2
Adhesion (OD450)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
1
2
DC-SIGN-Fc (ug/ml)
DC-SIGN
AZN-D1

Figure 32

A


Binding of HSV beads to K-DC-SIGN
60.00
50.00
40.00
30.00
20.00
10.00
0.00
% of binding
Control
AZN-D1
AZN-D2
HIV-1 gp120
HSV gB
HSV gC
HSV gE
viral proteins

B


HSVgB
60
50
40
30
20
10
0
Binding (%)
HSVgB
~
AZN-D1
mannan
EGTA